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3	Full name, address and postcode of the applicant	ML Laboratories 60 London Road St Albans AL 1NG UK
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4	Title of the invention	IMMUNOSUPPRESSION
5	Name of agent	HARRISON GODDARD FOOTE
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8	Statement of Inventorship Needed?	YES	
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	Description	~~28~~ ~~33~~ 29 ~~7~~	
	Claims	-	
	Abstract	-	
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10	Number of other documents attached		
	Priority documents		
	Translations of priority documents		
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	Other documents		
11	I/We request the grant of a patent on the basis of this application.		
	Signature	Harrison Goddard Foote	**22 October 1999**
12	Name and daytime telephone number of person to contact in the United Kingdom	**Dr Rob Docherty** **0113 225 8350**	

IMMUNOSUPPRESSION

1. FIELD OF THE INVENTION

This invention relates to immunosuppression and, more particularly, to immunosuppression in the context of xenotransplantation.

2. BACKGROUND TO THE INVENTION

Despite the established success of allogeneic organ transplantation, the increasing disparity between the supply and demand of organs must be overcome. Increasing the supply of allogeneic organs does not offer a satisfactory solution because even if all usable organs were transplanted this would still not meet the existing demand (1,2). This has led to a resurgence of interest in xenotransplantation (the transplantation of organs between animals of different species) as a viable and attractive alternative.

Xenotransplantation research has recently focused on the pig as a suitable animal donor in terms of size, physiological compatibility and breeding characteristics (3,4). Until recently however , discordant xenotransplantation has been limited by the inevitable occurrence of humorally-mediated hyperacute rejection (HAR) which rapidly triggers organ rejection upon revascularisation. HAR is the fate of most organs transplanted between discordant species. Recently, significant advances have been made in understanding the immunological basis of HAR, and many approaches have been employed to overcome it. Of significance, a variety of transgenic strategies are currently being employed including the expression of regulators of complement activity on porcine endothelial cells (5). It is foreseeable that short-term xenograft survival will soon be achieved (6). The recent advances in overcoming HAR have highlighted subsequent immunological barriers which must be surmounted to enable long-term xenograft survival. Both humoral and cellular arms of the immune response appear to play a role in the downstream events of immunological rejection. Clearly the most important of which is the existence of a formidable T cell mediated rejection response (7-11) previously obscured by the dominant role of HAR. *In vitro* , human T cells have been demonstrated

to play a central role in the recognition of xenogeneic cells (7,8,12) following sensitisation via the direct and indirect T cell activation pathways, which have been well documented for allorecognition and allograft rejection (13). Knowledge of the cellular mechanisms underlying allorejection has provided an important basis for the investigation of the T cell mediated xenoresponse.

At present, the major therapies to prevent cell mediated rejection of organ transplants rely on systemic immunonosuppressive drugs or monoclonal antibody (Mab) therapy directed against targets such as CD3, CD4, CD25, (14). Following reports that strong T cell xenoresponses can be generated *in vitro* (7,8,12), control of xenograft rejection may require levels of immunosuppresion much greater than the current standard doses. Such a strategy would not be desired in a xenograft context. Drugs must be taken for life, depress the entire immune system and result in an increased risk of infection and susceptibility to cancer (14). For the applicability of xenotransplantation to the clinic, targeting graft-specific strategies for tolerance induction/immunosuppression would clearly be highly advantageous. Whilst this has been difficult to achieve in an allotransplant context, xenotransplantation offers greater potential - with differences between species providing the option for the generation of reagents that are truly graft specific. In addition, there is the opportunity for the manipulation of both the porcine donor organ, and the human recipient's immune system, prior to transplantation (1).

3. DETAILED BACKGROUND

3.1 T cell activation and proliferation

Optimal proliferation of T cells, although initiated via ligation of the antigen specific CD3/TCR complex (Signal 1) requires additional costimulatory signals (Signal 2) (15,16,17) which are usually supplied by the antigen presenting cell (APC). Whilst antigenic stimulation of T cells in the presence of signal 2 induces T cell activation and proliferation (18), exposure of T cells to MHC-antigen complexes in their absence leads to aborted T cell proliferation and the development of clonal anergy (19,20). Manipulation of APC by aldehyde fixation (20,21) or heat treatment (19) has been

demonstrated to abrogate the ability of such cells to activate alloreactive T cells, without altering levels of MHC-II surface expression. Thus T cell receptor occupancy alone is insufficient to fully activate the T cell (17). Anergic T cells are best characterised by their lack of IL-2 production and their continued inability to produce IL-2 on subsequent exposure to antigen (22). Thus, confirming the two signal model of activation as predicted by Lafferty *et al* (23). For T cells to respond to a given antigenic stimulus, multiple activation signals are required from the APC (23).

The *in vivo* induction of T cell anergy in the absence of a secondary signal was first demonstrated by Jenkins and Schwartz in 1986 (24) using chemically fixed APC to present specific peptide to CD4 T helper clones. A multitude of *in vitro* and *in vivo* data has since been produced supporting the hypothesis that signal 1 in isolation fails to activate T cells (22), and that costimulatory signalling results from contact with other cells rather than via soluble factors. Fibroblasts transfected with human Class II MHC molecules, but not expressing the appropriate CS signals (lacking signal 2) can efficiently present antigen to class II restricted CD4 T cell clones, but these fail to cause antigen specific T cell proliferation, rendering cells anergic. The context in which T cells first encounter antigen therefore has an important bearing on subsequent immune responsiveness.

Thus, costimulatory molecules are essential for T cell activation and multiplication and result from interactions between receptors on T cells and their ligands expressed on the APC. The costimulatory signal itself, however, is neither antigen specific nor MHC restricted (25). In recent years the molecular interactions involved in mediating costimulation have been well defined. The two key pathways involve (i) B7-1, B7-2 (members of the B7 family) and (ii) CD40, which are expressed on the APC, and their counter-receptors CD28 and CD40 ligand (CD40L) respectively expressed on T cells. A large body of evidence, both *in vivo* and *in vitro* , clearly defines the crucial roles played by B7-1, B7-2 and CD40 in providing T cell costimulation (26-36). Furthermore, the simultaneous blockade of signalling via CD28-B7 and CD40-CD40L in an allotransplant

context prevented the onset of allograft rejection (37,38). *In vivo* , targeting the B7/CD28 interaction has been shown to prevent T cell sensitisation to graft antigen, thereby prolonging graft survival (38,39).

T cells can be sensitised against xenoantigens via one of two pathways - the direct and indirect pathways, which are analogous to the well documented T cell activation pathways against alloantigens (Figure 1). Direct recognition requires that the recipient T cells recognise intact xeno MHC-molecules complexed with peptide on donor stimulator cells. In contrast, indirect recognition requires that recipient APC process the xenoantigen prior to presentation to recipient T cells in the context of recipient MHC II. Self MHC II restricted T cells with specificity for the xenoantigen will recognise the peptide and respond. Whilst the majority of data reported is of indirect xenorecognition responses, cell mediated rejection via the direct route has also been documented (7,8,9,11,12,40,41,42). Vigorous human T cell proliferative responses directed against porcine tissues *in vitro* have been documented from studies both in this laboratory and others.

3.2 Costimulatory molecules

The crucial role played by costimulatory molecules in determining the result of TCR-CD3 receptor engagement with MHC and peptides has been demonstrated extensively both *in vivo* and *in vitro* . Anti-costimulatory molecule strategies aimed at either the receptors or their ligands are being used as therapeutic strategies for altering the immune response. Such approaches have been tested in model transplant systems to alter cell mediated responses thereby preventing graft rejection (14,37,38,43-47).

B7-1 (B7/BB1, CD80) and B7-2 (CD86) both belong to the Immunoglobulin superfamily and are heavily glycosylated transmembrane proteins (25). B7-1, a B cell activation molecule was first identified in 1989 (27), followed by B7-2 in 1993 (49). Both human B7-1 and B7-2, and the murine homologues have now been cloned and functionally characterised (25) . B7-1 and B7-2 are constitutively expressed on splenic and blood

dendritic cells and are induced on B cells and monocytes upon activation (34,50,). B7-1 and 2 are highly homologous and are the natural ligands for the T cell antigen CD28 (50). Cytotoxic T lymphocyte antigen-4 (CTLA-4), a cell surface glycoprotein has been identified as a second receptor for the B7 family of molecules (51) and is homologous to CD28 with 31% sequence identity. Both B7 isoforms bind to CTLA-4 with higher affinity than to CD28 (30,50,52). Whilst CD28-B7 receptor engagement results in an APC-derived costimulatory signal involved in antigen specific IL-2 production both *in vivo* and *in vitro* (53,54), CTLA4 appears to function as a negative regulator of T cell activation (55, 56, 57). Cross-linking by anti-CTLA4 antibodies has been demonstrated to antagonise CD28 ligation (58) and, in addition, CTLA4 knock-out mice die due to uncontrolled lymphocyte proliferation within the first few weeks of life (59). Thus, CTLA4 ligation is thought to be crucial for the maintenance and regulation of immune responses. The underlying mechanisms have not, however, been clearly defined.

Among costimulatory molecules, the B7 family appears to be unique, since ligation by CD28 of either B7-1 or B7-2 is both necessary and sufficient to prevent the induction of anergy (34). The CD28-B7 interaction is thought to deliver crucial signals to sustain proliferation of activated T cells. These observations are supported by *in vitro* data showing that whilst cells deficient in B7 fail to stimulate a primary MLR, transfectants expressing high levels of B7 gained the capacity to stimulate the production of IL-2 by alloreactve T cells and to co-stimulate a polyclonal population of purified T cells cultured with immobilised anti-CD3 Mab (31). Artificial APC generated by stably transfecting NIH-3T3 cells with HLA-DR7, B7 or both, clearly demonstrated that following presentation of tetanus toxoid (TT) optimal T cell proliferation and IL-2 production resulted only when both molecules were present. In the absence of B7, clonal anergy resulted (58).

Porcine B7-2 (PoB7-2) has been cloned from aortic endothelial cells (60). Following transient transfection of porcine B7-2, human umbilical vein endothelial cells strongly costimulated IL-2 production by human T cells. This costimulation of human T cells by

poB7-2 was shown to be as effective as costimulatory signals provided by human B7-1 or B7-2 and could be specifically blocked by huCTLA4Ig. Thus poB7-2 strongly contributes to the immunogenicity of porcine endothelium (60).

Although B7-1 and B7-2 mediated interactions appear to be central to the development of T cell specific immunity, additional costimulatory pathways of importance exist. The most crucial of which involves the CD40 and CD40 ligand (CD40L) interaction (34).

CD40 is a 50kDa surface glycoprotein belonging to the TNF-receptor superfamily. CD40 is expressed on various APC including among others, monocytes, dendritic cells and activated macrophages. Other cell types including endothelium also express CD40 (34). Its counter-receptor CD40L (CD154, gp39, TRAP) is a 33 kDa type II integral membrane protein (34,36) transiently expressed on activated CD4 T cells. The CD40-CD40L interaction has been demonstrated to play an important role in both the humoral and cellular arms of the immune response with a dominant role in B cell activation. Whilst cross linking of CD40 on B cells is essential for B cell growth and isotype switching, it also results in the upregulation of B7 expression (50). Levels of B7 expression (and thus APC capacity) of monocytes and dendritic cells are clearly unregulated following CD40 signalling (34). Data from CD40 knock-out mice demonstrated that CD40L signalling following ligation by CD40 plays an important role in T cell activation (61). Transfection of the murine P815 mastocytoma cells with CD40 (or B7-1) enabled previously non-stimulatory P815 cells to mediate the costimulation necessary for polyclonal T cell activation and the generation of cytokines (34). CD40-CD40L interactions have also been demonstrated to play a critical role in allograft rejection (62,63).

Resting B cells do not normally express B7-1/B7-2 at high levels until they are activated (50). Activation of B cells following simultaneous engagement of MHC-peptide/TCR and CD40-CD40L leads to the upregulation of B7 family members on B cells, thereby enhancing the stimulation and subsequent activation of T cells (34,36). Thus, the CD40-CD40L interaction influences costimulatory activity by inducing expression of the

B7 family of molecules and perhaps other costimulatory molecules, thereby playing a key role in T cell activation . The clear synergistic effects of CD40 and B7 indicate the importance of both costimulatory pathways for the initiation and amplification of T cell dependent immune responses (38). CD40-CD40L interactions have also been shown to play a crucial role in the generation of cytotoxic T lymphocyte (CTL) responses by modifying the functional status of a dendritic cell (64,65,66)

Extensive studies have demonstrated the importance of blocking B7-CD28 and/or CD40-CD40L interactions in the context of both allo and xenotransplantation. Data strongly supporting this includes the use of CTLA4Ig to block signalling via CD28-B7 resulting in enhanced graft survival and the prevention of chronic rejection in a rat cardiac allograft model (44,45) and a murine aortic allograft model (43). In these models, administration of CTLA4Ig caused partial (44) or complete (46) tolerance to graft antigen by inducing T cell anergy. Treatment of allo pancreatic islet transplants with anti-B7-2 and B7-1 antibody has also been demonstrated to inhibit transplant rejection (14). Similar results were obtained in models inhibiting CD40 signalling in a mouse cardiac allotransplant models (37,47,62). Two studies detailing the simultaneous blockade of signalling via CD28-B7 and CD40-CD40L prevented the onset of allorejection. Concurrent prolonged inhibition of both pathways completely abrogated the onset of chronic rejection in a mouse allo model (37) and in a skin and heart allo model (38).

In the realm of xenotransplantation, Lenshow and colleagues have, demonstrated long-term donor specific tolerance of human islets transplanted into mice with concomitant treatment with CTLA4Ig (46). Graft specific tolerance was demonstrated to be a direct consequence of inhibiting recognition via B7 expressing APC. In addition, Tran *et al* (67) demonstrated short term suppression with CTLA4-Fc treatment. There is limited data available on the simultaneous blockade of both pathways in the xenotransplantation context, with the prolonged survival of rat and porcine skin transplanted into murine recipients (63).

In vitro and *in vivo* data have clearly demonstrated that targeting the interactions mediated by either the CD28-B7, CD40-CD40L, or both pathways has prevented the sensitisation of T cells to alloantigen and xenoantigen from engrafted tissue thereby prolonging graft survival ().

3.3 Peptide immunisation strategy

Previous *in vivo* studies using synthetic peptides conjugated to carrier molecules as immunogens have demonstrated the ability to generate the production of biologically active antibodies (68). There is now an extensive literature detailing peptide immunisation strategies which demonstrate enhancement of antibody production by carrier presentation(68-72). Thus, appropriate T cell epitopes can be used to prime T cells for subsequent help to B cells. Recent data has been published reporting the production of IgG by self-reactive B cells following immunisation with a self reacting antigen covalently coupled to a carrier molecule (70). Thereby demonstrating that B cell tolerance to self protein can be overcome.

As mentioned above, in order to be recognised by T cells, antigen (self or foreign) must be processed and presented by APC. B cells can act as highly potent APC following endocytosis of antigen via IgG receptors . In the presence of a full complement of activation signals (TCR engagement plus costimulation) T cell activation will occur resulting in the subsequent generation of antibody.

Peptides from self proteins are processed and presented to T cells in the same manner as foreign proteins, but because of T cell tolerance, presentation of self peptides does not normally result in T cell activation (70). The absence of T cell recognition may therefore explain, in part, why potentially reactive B cells fail to respond.

The ability to overcome B cell non-responsiveness to self peptides has recently been demonstrated by Dalum *et al* (69). An autoantibody response was generated by the provision of additional T cell help in the form of a strong foreign carrier T cell epitope.

Further studies have demonstrated that synthetic peptides conjugated to T cell carrier molecules are capable of overcoming B cell non-responsiveness if significant numbers of self-reactive B cells are present in the host (69,70). Insertion of a single foreign T cell epitope into the sequence of Ubiquitin, elicited strong autoantibody production directed against the native molecule (69). In an elegant study by Sad, using GnRH as a self protein chemically linked to diphtheria toxoid (DT) as the synthetic T cell epitope, autoantibodies were produced with specificity for native GnRH (71,72). Following the initial vaccination, the continued presence of the native GnRH *in vivo* maintained the production of Ab. Continued antibody production caused sterility in the immunised mice due to the sustained anti-GnRH antibody response maintained by the continued presence of the native molecule against which the specific B cells were producing antibody. The DT carrier provoked a helper T cell response to assist GnRH specific B cells and break B cell tolerance.

4. STATEMENTS OF INVENTION

The present invention involves the use of a foreign T cell carrier to exert significant influences on subsequent responses to molecules conjugated to the carrier. By such means autoantibody responses may be directed against costimulatory molecules in a xenotransplantation context.

According to the present invention there is provided a method of improving the tolerance of an animal, including a human being, to a xenograft, the animal having T cell mediated immunity, the method comprising causing the animal to raise an antibody against a xeno-molecule involved in the generation of a rejection response in the animal, said antibody being raised by immunising the animal with a chimeric peptide comprising a T cell epitope against which the animal has immunity and a B cell epitope of said xenomecuoe.

Accordingly, xenograft specific tolerance is induced in transplant recipients by targeting the direct T cell mediated response by the use of chimeric peptide constructs to stimulate the generation of specific anti-graft tolerance-promoting antibodies by the recipient prior

to transplantation. By way of example, the chimeric peptides comprise a T cell epitope conjugated to sequences of porcine costimulatory molecules, B7-1, B7-2 and CD40. The presence of the engrafted tissue will then serve to maintain and perpetuate the production of antibody by the recipient's B cells.

The present invention also provide a chimeric peptide comprising a T cell epitope and a B cell epitope, said T cell being that of an animal, including a human being of a first species and said B cell being of an animal of a second species, said first and second species such that xeno transplantations suitable from an animal of said second species to an animal of said first species.

In addition, the present invention provides the use of a chimeric peptide improving the tolerance of an animal, including a human being, to a xenograft, the chimeric peptide being as defined above.

According to a further aspect of the invention there is provided an immunogenic composition comprising at least one peptide antigen capable of inducing T- cell immunity to at least the effective part of at least one porcine co-stimulatory molecule involved in the activation of at least one T- cell.

In a preferred embodiment of the invention said immunogenic composition comprises at least one peptide antigen derived from at least one of; CD40; CD40L; B7.1; B7.2;. Preferably said peptide antigen is derived from B7.2. Ideally said peptide is derived from the amino- terminal domain of porcine B7.2, or at least that part of the amino terminal domain that is exposed at the cell surface of a cell presenting B7.2. More ideally still said peptide antigen is selected from the peptide sequences presented in Table 1.

Ideally said peptide antigen is;
ISQAVHAAHAEINEAGRCSSTQGYPEPQR (peptide 6).

More ideally still, said peptide antigen is;

ISQAVHAAHAEINEAGRGLVPIHQMS (peptide 4).

Preferably, said peptide antigen comprises at least 9 amino acid residues. More ideally still said peptide comprises 10 – 30 amino acid residues.

It is well known in the art that peptide antigens presented by major histocompatibility complex (MHC) molecules on antigen presenting cells ideally comprise 9 or 10 amino acid residues.

According to a further aspect of the invention there is provided an immunogenic composition according to any previous aspect or embodiment of the invention wherein said composition further comprises at least one agent capable of enhancing T- cell immunity.

In a preferred embodiment of the invention said agent is an adjuvant.

It is well known in the art that adjuvants are useful in promoting immune responses to selected antigens. These adjuvants are either crosslinked or coupled to the antigen or co-administered to the animal with the antigen. Adjuvants useful in promoting immune responses are detailed in Vaccine Design:The Subunit and Adjuvant Approach Chapter 7, p141- 228, Plenum Press, New York, 1995. Various carriers, excipients or diluants are available in which said immunogenic composition can be stored and/or administered. For example, and not by way of limitation, the encapsulation of the immunogenic composition in liposomes is a conventional practice. Liposomes are phospholipid based vesicles which are useful as carrying agents for immunogenic compositions and the like.

In a further preferred embodiment of the invention said adjuvant is at least part of the ovalbumen polypeptide. More preferrably still said adjuvant is at least part of the tetanus toxoid polypeptide.

According to yet a further aspect of the invention there is provided an antibody, or at least the effective part thereof, directed to at least one region of at least one porcine co-stimulatory molecule. Ideally said antibody is specific for porcine co-stimulatory molecules.

Ideally said antibody is directed to at least one region involved in the interaction between co-stimulatory molecules and thereby inhibits the ligation of said co-stimulatory molecules which results in T- cell activation.

In a preferred embodiment of the invention said antibody is a monoclonal antibody, or at least the effective part thereof.

It will be apparent to one skilled in the art that antibodies according to the invention will have utility with respect to monitoring the expression of porcine co-stimulatory molecules presented by porcine tissues/organs.

According to yet a further aspect of the invention there is provided a method to improve the tolerance of an animal to a xenograft comprising:

i) administering at least one immunogenic composition according to any previous aspect or embodiment of the invention to an animal; optionally
ii) monitoring the immune status of said animal to said immunogenic composition;
iii) transplantation of at least one porcine tissue/organ into said animal; and, optionally
iv) monitoring the animal for a rejection response to said porcine tissue/organ.

In a preferred method of the invention said animal is human.

It will be apparent to one skilled in the art that (ii) above can be conducted either by monitoring for the presence of antibodies to co-stimulatory molecules in sera (for example by ELISA or by FACS analysis of cells expressing said co-stimulatory molecules), or alternatively, or in addition, monitoring the presence of cytolytic T- cells in the blood of the treated animal by conventional T- cells lysis assays.

The potential benefits of the use of a chimeric peptide of the invention are that it avoids the need for injection of blocking antibodies or fusion proteins. Furthermore, the induction of a recipient antibody response circumvents the problems most commonly associated with administration of xenogeneic antibodies or fusions proteins, namely the immune response against the administered reagent.

5. SPECIFIC EMBODIMENTS

5.1 Cloning porcine costimulatory molecules

5.1.1 Cloning porcine B7-2

RNA was extracted from primary and transformed porcine cells using a standard protocol. mRNA was then reverse transcribed and porcine B7-2 (poB7-2) amplified from the cDNA by 35 cycles of PCR at 56^0 C with 1.5mM magnesium. The 5' and 3' primers GCATGGATCCATGGGACTGAGTAACATTCTCTTTG and GCATGTCGACTTAAAAATCTGTAGTACTGTTGTC respectively were designed on the basis of the published poB7-2 sequence (60) to overlay the start and stop codons (Figure 2). A 956 base pair fragment was generated and subcloned into the BamH1 & Sal1 restriction sites of pbluescript. The nucleotide sequence was determined using standard m13 forward and reverse primers. The sequence of a single clone, CD86(i) is illustrated in Figure 3, with comparison to the published sequences from porcine (Figure 4), human and murine B7-2 (Figure 5). One base pair difference is detected between our clone, CD86(i), and the published sequence at the 3' prime end. This, however, is unlikely to be an important difference with respect to either poB7-2 expression or binding

to its ligand. The predicted amino acid sequence of CD86(i) , compared to that of porcine, human and mouse B7-2 is shown in Figure 6.

5.1.2 Cloning porcine B7-1 and CD40

RNA extracted from phytohaemagglutinin (PHA) or poke-weed mitogen (PMW) stimulated porcine PBMC and transformed porcine endothelial cells is being used to amplify cDNA encoding the costimulatory molecules B7-1 and CD40. B7-1 Primers were designed on the basis of conserved areas following comparison of murine and human (29,49) sequences. External (lying outside the coding region) AGACCGTCTTCCTTTAG(3'i), TTGGATCCTCCATGTTATCCC (3'ii) and AGCATCTGAAGC (5') and internal (within the coding region) ATGGATCCTCCATTTTCCAACC (3') and TTGTCGACATCTACTGGC (5') primers have been designed as depicted in Figure 7. The generation of two 3' primers is due to significant differences between the human and murine sequences in the terminal coding regions. Resulting PCR fragments will be subcloned as described above using the restriction sites BamHI and SalI contained within the promoter sequence. Constructs will then be sent for sequence confirmation.

CD40 primers were designed in a similar manner following sequence alignment of published CD40 sequences from human, mice and cattle (73,74,75) as illustrated in Figures 8A & B. The 5' and 3' primer sequences are GGATCCTCACTGTCTCTCCTGCACTGAGATGCGACTCTCCTCTTTGCCGTCCG TCCTCC and GAATTCATGGTTCTGTTGCCTCTGCAGTG respectively containing the BamHI and EcoRI restriction sites.

5.2 Generation of porcine costimulatory molecule expressing cell transfectants

The poB7-2 molecule (CD869(i)) has been subcloned into the eukaryotic expression vector pci.neo carrying the neomycin drug-selectable marker. This is being used to transfect M1 and M1.DR1 transformed murine cell lines using a standard calcium

phosphate precipitation method. G418 resistant pci.neo expressing cells will be selected using dynabead purification and highly expressing clones is selected by limiting dilution.

Stable poB7-2 M1 and P815 transfectants have been generated by this approach using the poB7-2 DNA construct supplied to us by Maher *et al* (Figure 9). transient transfections of M1 and P815 cells have been generated using our CD86(i) construct (Figure 10).

3 particular assays are undertaken using the CD86(i) transfected cells.

(I) comparative costimulatory function of poB7-2 with human B7-1 in the context of MHC restriction;

(II) flow cytometric analysis of specific anti-poB7-2 antibodies in the sera of immunised mice; and

(III) generation of specific anti-poB7-2 monoclonal antibodies.

(I) Comparative *in vitro* analysis is performed to determine the costimulatory function of poB7-2 or poB7-1 in the context of the human MHC class II molecule HLA-DR1, with that of human B7-1 or B7-2 in the context of DR1, in proliferation assays with human or porcine responders.

(II) Transfected P815 cells are crucial reagents for the detection of porcine anti-B7-2 antibody in the sera of immunised mice which have undergone the chimeric peptide immunisation regimen. Flow cytometric analysis with control or poB7-2 -transfected P815 cells, reflects the specificity of sera for B7-2. Preliminary studies with C57BL-6 mice immunised with a pool of all nine B7-2 peptides have demonstrated the preferential binding of B7-2 peptide sera to porcine B7-2 transfected P815 cells (Figure 11a and 11b).

(III) Mab with specificity for poB7-2 are generated by immunisation of Balb/c mice with poB7-2 expressing P815 cells . The spleens from immunised mice are fused with the NS0 fusion partner and successful fusion's selected by virtue of HAT selection. Flow cytometric staining of poB7-2 P815 transfectants with culture supernatants enable the identification of MAb secreting cells. Cells are grown in culture and the medium

harvested for antibody purification by passage over Protein G following ammonium sulphate precipitation. Techniques for the preparation on monoclonal antibodies are well known in the art and with reference to publications such as Harlow and Lane Antibodies; A Laboratory Manual; Cold Spring Harbour Laboratories.

MAb with specificity for B7-1 and CD40 are generated using the same protocol. These MAb will provide valuable reagents for further characterising the expression of CS molecules on relevant porcine tissues.

5.3 Design and synthesis of poB7-2/OVA chimeric peptide constructs

Nine different peptides derived from the sequence of poB7-2 were initially selected for synthesis. Porcine B7-2 peptides, 6-22mer in size, were selected as determined by the predicted size of a B cell epitope. Peptides were selected for synthesis in combination with a T cell epitope OVA 323-339. B7-2 peptides were selected on the basis of 3D computer modelling (in collaboration with Paul Travers) and on the basis of predicted antigenicity and hydrophilicity using the SeqAid II computer software package. All of the nine peptides reflect linear epitopes. The positions of the nine peptides in the cloned poB7-2 sequence are indicated (Figure 12). Synthetic peptide sequences are detailed in Table 1

Table 1

Peptide Name	Peptide Sequence	Position
Peptide 1	ISQAVHAAHAEINEAGRSFDQATWTLR	81-90
Peptide 2	ISQAVHAAHAEINEAGRLPCHFTNSQ	32-40
Peptide 3	ISQAVHAAHAEINEAGRKGPHGLVPIHQMS	109-121
Peptide 4	ISQAVHAAHAEINEAGRGLVPIHQMS	113-121
Peptide 5	ISQAVHAAHAEINEAGRVQIKDKGSYQC	94-104
Peptide 6	ISQAVHAAHAEINEAGRCSSTQGYPEPQR	151-162
Peptide 8	ISQAVHAAHAEINEAGRKSQAYFNETGEL	21-32
Peptide 9	ISQAVHAAHAEINEAGRASLKSQAYFNET	17-29
Peptide 10	ISQAVHAAHAEINEAGRYMGRTSFDQATWT	76-88
Ova Peptide	ISQAVHAAHAEINEAGR	323-339

The peptide sequences and amino acid positions for peptides 1-10 relate to the position of the B7-2 peptide sequence within porcine B7-2. The amino acid position for the ova sequence is only indicated for the Ova peptide. A 17 amino acid peptide from chicken egg albumin (ovalbumin) was selected as the T cell epitope, OVA323-339 (ISQAVHAAHAEINEAGR). This epitope was selected on the basis of published reports for the generation of a H-2^b restricted T cell response (76,77).We have demonstrated the ability of C57BL-6 mice (H-2^b haplotype) to mount a proliferative response to both the native molecule and to the OVA 323-339 peptide following immunisation with whole ovalbumin (Figure 13). Peptides were generated on a peptide synthesiser (Genosys) and crude peptides were purified by HPLC to greater than 70% purity. Sera from OVA control immunised mice should ideally not recognise the 323-339 sequence, indicating that the T cell epitope is devoid of B cell determinants.

5.4 Tolerance induction

5.4.1 *In vivo* tolerance induction strategy

C57BL-6 mice are immunised with whole ovalbumin in CFA, followed by either control peptide (OVA peptide) or CS peptides (OVA-B7-2 constructs) for three weekly immunisations. Blood is collected following sacrifice and sera prepared using a standard

technique. Presence of specific mouse anti-porcine B7-2 IgG and/or IgM Ab is detected by one of two strategies.

Peptide ELISAs are used to screen for the presence of anti-peptide antibody in the sera. Peptides are coated to plates by virtue of aldehyde linkages to allow free access of Ab to the peptide (78), Plates are coated with individual peptides or the ova control peptide to enable the identification of specific peptides of interest. To detect reactivity of sera with the native B7-2 molecule expressed on the surface of PoB7-2 transfected P815 cells, flow cytometry is performed following surface staining. Having identified CS peptide of interest (peptide ELISA positive and recognising native B7-2) the sera is used to inhibit *in vitro* T cell proliferative responses. This determines whether the antibody is a blocking antibody.

In vivo studies are performed using the islet transplant system. Antibodies which recognise the native molecule but fail to block a proliferative response are useful polyclonal antibody reagents.

Immunisations involved two groups of mice, one received a pool of all nine B7-2 peptides, and one receiving ova control peptide. The harvested sera were screened by peptide ELISA (Figure 14a or 14b) which enabled the identification of peptides of interest. Antisera to peptides 2, 4 and 6 clearly demonstrate preferential binding to B7 peptide than to ova control. The sera has also demonstrated enhanced binding to poB7-2 transfected cells (Figure 11). Peptide 4 and 6 were selected as candidate peptides and used in subsequent immunisation protocol. Immunisation with peptide 4 or 6 clearly produced a significant level of IgG with specificity for peptides 4 and 6 in the sera of immunised mice (Figure 15a and 15b). The specificity of the sera for peptide 4 and not to ova control is demonstrated in Figure 16. The ability of sera from peptide 4 and 6 immunised mice to specifically recognise the native porcine B7-2 molecule expressed on the surface of porcine B7-2 transfected P815 cells is illustrated in Figure 17a and 17b. Untransfected control P815 cells do not stain with the Peptide 4 or 6 sera, neither do

control or transfected cells incubated with ova peptide sera. Similar protocols will be followed with peptide 2. These data clearly demonstrate the ability of this technique to generate anti-peptide antibody directed against an amino acid sequence, by virtue of a carrier T cell epitope.

An identical strategy will be followed with peptides designed on the basis of porcine CD40 and porcine B7-1 once the DNA sequence encoding these molecules has been elucidated.

5.4.2 Functional assessment; prolongation of pancreatic islet xenograft survival

Islet xenografts being non-vascular are rejected solely by T cell mediated mechanisms (79,80), thereby providing an ideal system to study modulation of T cell mediated reactions, please see Figure 18. A very clear role for cell mediated rejection of islets has been demonstrated and is reported to be greater than the comparable alloresponse (80). Transplantation of porcine pancreatic islets to mice is an established procedure, which is well documented in the literature (80-83). Studies within this laboratory have demonstrated a decrease in hyperglycaemia (Figure 18) following transplantation of pancreatic islets from large white pigs under the kidney capsule of C57BL-6 mice rendered diabetic by intraperitoneal administration of streptozotocin, please see Figure 19 and 20. Further optimisation of the isolation procedure (84,85) is required to enable purification of fully functional islets. Transplanted islets usually survive between 6-10 days in the absence of any immunosupression. Successful modulation of direct T cell mediated xenorejection will be monitored by prolongation of islet survival beyond day 10, with comparison to the appropriate controls.

The results obtained with B7-2 to date, demonstrate the ability of synthetic B7-2 peptides conjugated to a known T cell helper epitope to generate the production of anti-pocine B7-2 antibody *in vivo*. These antibodies if directed towards the binding site between B7 isoforms and CD28, in association with antibodies directed against CD40-CD40L will

block the costimulation of human T cells with direct anti-pig xenoreactivity thereby prolonging islet survival in a xenotransplantation context.

Having established the suitability of such an approach in a pig islet to mouse *in vivo* model, studies would progress to pig to primate transplantation systems prior to clinical trials.

5.5 Adaptations for clinical use of these strategies

For clinical applicability the following requirements are necessary:

(I) selection of a suitable T cell epitope to replace OVA. One candidate molecule is tetanus toxiod (TT) which is a widely used antigen for use in human immunisation strategies (68,86). The prior immunisations of most adults with TT is an additional benefit to this strategy as memory T cells are already present in the circulation.

(ii) An efficient and rapid screening method is used to detect the presence of anti-donor (pig) B7-2 antibodies in the absence of a specific B7-2 directed T cell response generated by the recipient which would accelerate graft rejection.

6. SUMMARY OF SPECIFIC EMBODIMENTS

The above examples relate to a novel strategy to inhibit costimulation by porcine cells of human T cells with direct anti-pig xenoreactivity. This is of particular importance in the context of xenotransplantation of porcine organs due to the expression of costimulatory molecules on porcine endothelial, as well as bone marrow-derived antigen presenting cells.

Recipients are immunised with hybrid synthetic peptides comprising a T cell epitope conjugated to sequences of the porcine costimulatory molecules, CD80, CD86 and CD40. Peptides that induce antibodies specific for regions of the costimulatory molecules involved in binding to their counter-receptors on human cells (CD28 and CD154) are therefore capable of blocking the delivery of costimulation. Once the antibody response has been induced, the transplanted organ will recall this response due to the expression of

the costimulatory molecules, thereby sustaining this response, and providing an endogenous mechanism of costimulatory blockade.

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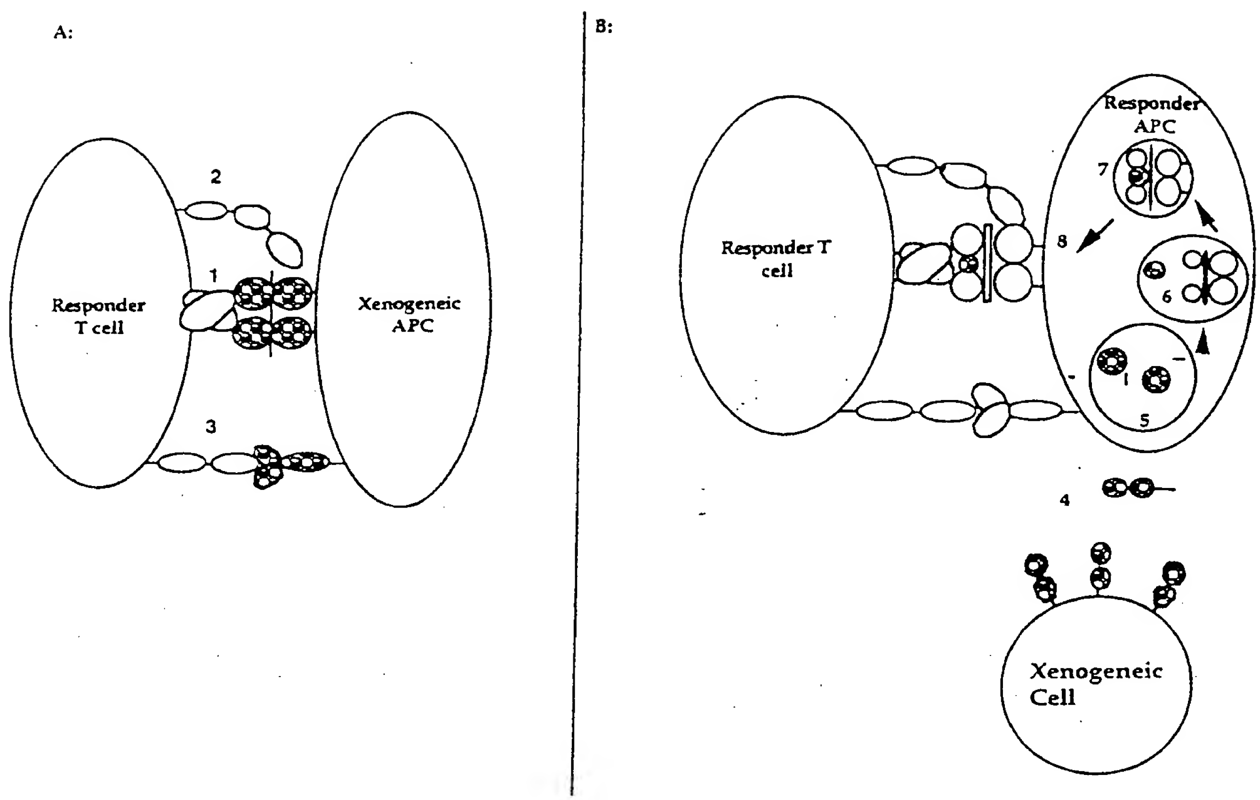
Figure 1



A; Diagrammatic representation of direct xenorecognition.
T[illegible]es of molecular interactions necessary for efficient direct xenorecognition are numbered 1 - 3.
Cognate interaction between TCR on responder T cell and MHC molecules on xenogeneic antigen presenting cells.
2 Non cognate interaction between co-receptors CD4 and membrane proximal domains of MHC class II, and CD8 and $\alpha 3$ domains of MHC class I.
3 Non cognate interactions between accessory and costimulatory molecules. Important interactions are between B7 family (APC) and CD28 (T), LFA-3 (APC) and CD2 (T), and ICAM-1 (APC) and LFA-1 (T)

B; Diagrammatic representation of indirect xenorecognition
Xenoantigens (4), released by xenogeneic cells, are taken up and processed (5) into peptide fragments by specialised antigen presenting cells (6) before binding to MHC class II molecules (7) and display on the cell surface (8) for presentation to xenospecific self-class II MHC-restricted T cells.

Figure 1: Diagramatic comparison of direct and indirect xenorecognition pathways.

GCATGGATCCATGGGACTGAGTAACATTCTCTTTG

1 ATGGGACTGAGTAACATTCTCTTTGTGATGGTCCTCCT

[illegible] GCTCTCTGGTGCTGCCTCCTTGAAAAGTCAGGCATATTTCAATGAGA

86 CTGGAGAACTGCCGTGCCATTTTACAAACTCGCAGAACCTAAGCCTG

133 GATGAGCTGGTCATATTTTGGCAGGACCAGGATAACCTGGTTCTCTA

181 CGAGCTATACCGAGGCCAAGAGAAGCCTCATAATGTTAATTCCAAG

227 TATATGGGTCGCACAAGCTTTGACCAGGCCACCTGGACCCTGAGACT

274 CCACAACGTTCAAATCAAGGACAAGGGCTCATATCAATGTTTCATC

321 CATCATAAAGGGCCGCATGGACTTGTTCCTATCCACCAGATGAGTTC

[illegible]3 TGACCTATCATTGCTTGCTAACTTCAGTCAACCTGAAATAAACCTAC

415 TTACTAATCACACAGAAAATTCTGTCATAAATTTGACCTGCTCATCT

462 ACACAAGGCTACCCAGAACCCCAGAGGATGTATATGTTGCTAAATA

509 CGAAGAATTCAACCACTGAGCATGATGCTGACATGAAGAAATCTCA

556 AAATAACATCACGGAACTCTACAATGTATCAATCAGGGTGTCTCTT

602 CCCATCCCTCCCGAGACAAATGTGAGCATCGTCTGTGTCCTGCAACTT

649 GAGCCAAGCAAGACACTGCTTTTCTCCCTACCTTGTAATATAGATGC

696 AAAGCCACCTGTGCAACCCCCTGTCCCAGACCACATCCTCTGGATTGC

743 AGCTCTACTTGTAACAGTGGTCGTTGTGTGGGATGGTGTCCTTTGT

790 AACACTAAGGAAAAGGAAGAAGCAGCCTGGCCCCTCTAATGA

837 ATGTGGTGAAACCATCAAAATGAACAGGAAGGCGAGTGAACAAAC

884 TAAGAACAGAGCAGAAGTCCATGAACGATCTGATGATGCCCAGTGT

931 GATGTTAATATTTTAAAGACAGCCTCAGAT**GACAACAGTACTACAG**

GACAACAGTACTACAG

978 ATTTTTAATTAAAGAGTAAACTCC

ATTTTTAAGTCGACATGC

Figure 2: Position of 5' and 3' primers (highlighted in bold type)with respect to the published coding sequence of porcine CD86. The underlined sequences ATG and TAA represent the start and stop codons respectively.

```
   1  CACCGCGGTG CGGCCGCTCT AGAACTAGTG GATCCATGGG ACTGAGTAAC
  51  ATTCTCTTTG GGATGGTCCT CCTGCTCTCT GGTGCTGCCT CCTTGAAAAG
 101  TCAGGCATAT TTCAATGAGA CTGGAGAACT GCCGTGCCAT TTTACAAACT
 151  CGCAGAACCT AAGCCTGGAT GAGCTGGTCA TATTTTGGCA GGACCAGGAT
 201  AACCTGGTTC TCTACGAGCT ATACCGAGGC CAAGAGAAGC CTCATAATGT
 251  TAATTCCAAG TATATGGGTC GCACAAGCTT TGACCAGGCC ACCTGGACCC
 301  TGAGACTCCA CAACGTTCAA ATCAAGGACA AGGGCTCATA TCAATGTTTC
 351  ATCCATCATA AAGGGCCGCA TGGACTTGTT CCTATCCACC AGATGAGTTC
 401  TGACCTATCA GTGCTTGCTA ACTTCAGTCA ACCTGAAATA AACCTACTTA
 451  CTAATCACAC AGAAAATTCT GTCATAAATT TGACCTGCTC ATCTACACAA
 501  GGCTACCCAG AACCCCAGAG GATGTATATG TTGCTAAATA CGAAGAATTC
 551  AACCACTGAG CATGATGCTG ACATGAAGAA ATCTCAAAAT AACATCACGG
 601  AACTCTACAA TGTATCAATC AGGGTGTCTC TTCCCATCCC TCCCGAGACA
 651  AATGTGAGCA TCGTCTGTGT CCTGCAACTT GAGCCAAGCA AGACACTGCT
 701  TTTCTCCCTA CCTTGTAATA TAGATGCAAA GCCACCTGTG CAACCCCCTG
 751  TCCCAGACCA CATCCTCTGG ATTGCAGCTC TACTTGTAAC AGTGGTCGTT
 801  GTGTGTGGGA TGGTGTCCTT TGTAACACTA AGGAAAAGGA AGAAGAAGCA
 851  GCCTGGCCCC TCTAATGAAT GTGGTGAAAC CATCAAAATG AACAGGAAGG
 901  CGAGTGAACA AACTAAGAAC AGAGCAGAAG TCCATGAACG ATCTGATGAT
 951  GCCCAGTGTG ATGTTAATAT TTTAAAGACA GCCTCAGATG ACAACAGTAC
1001  TACAGATTTT TAAGTCGACC TCGAGGGGGG GCCCGGTACC AGCTTTTGTT
```

Figure 3: Nucleotide sequence of CD86(i) obtained by RT-PCR amplification of cDNA extracted from a transformed porcine endothelial cell line A8.

Figure 4: Comparison of the nucleotide sequence of CD86(i) with the published sequence for porcine CD86.

Figure 4: 5/32

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                               10        20        30        40
                               |         |         |         |
                               ATGGGACTGAGTAACATTCTCTTTGTGATGGTCCTCCTGCTCTCTGG
                               ........................ .......................
CACCGCGGTGCGGCCGCTCTAGAACTAGTGGATCCATGGGACTGAGTAACATTCTCTTTGGGATGGTCCTCCTGCTCTCTGG
        |         |         |         |         |         |         |         |
        10        20        30        40        50        60        70        80

 50        60        70        80        90        100       110       120
 |         |         |         |         |         |         |         |
TGCTGCCTCCTTGAAAAGTCAGGCATATTTCAATGAGACTGGAGAACTGCCGTGCCATTTTACAAACTCGCAGAACCTAAGC
..................................................................................
TGCTGCCTCCTTGAAAAGTCAGGCATATTTCAATGAGACTGGAGAACTGCCGTGCCATTTTACAAACTCGCAGAACCTAAGC
      |         |         |         |         |         |         |         |
      90        100       110       120       130       140       150       160

 130       140       150       160       170       180       190       200       210
 |         |         |         |         |         |         |         |         |
CTGGATGAGCTGGTCATATTTTGGCAGGACCAGGATAACCTGGTTCTCTACGAGCTATACCGAGGCCAAGAGAAGCCTCATA
..................................................................................
CTGGATGAGCTGGTCATATTTTGGCAGGACCAGGATAACCTGGTTCTCTACGAGCTATACCGAGGCCAAGAGAAGCCTCATA
     |         |         |         |         |         |         |         |
     170       180       190       200       210       220       230       240

         220       230       240       250       260       270       280       290
         |         |         |         |         |         |         |         |
ATGTTAATTCCAAGTATATGGGTCGCACAAGCTTTGACCAGGCCACCTGGACCCTGAGACTCCACAACGTTCAAATCAAGGA
..................................................................................
ATGTTAATTCCAAGTATATGGGTCGCACAAGCTTTGACCAGGCCACCTGGACCCTGAGACTCCACAACGTTCAAATCAAGGA
   |         |         |         |         |         |         |         |
   250       260       270       280       290       300       310       320

       300       310       320       330       340       350       360       370
       |         |         |         |         |         |         |         |
CAAGGGCTCATATCAATGTTTCATCCATCATAAAGGGCCGCATGGACTTGTTCCTATCCACCAGATGAGTTCTGACCTATCA
..................................................................................
CAAGGGCTCATATCAATGTTTCATCCATCATAAAGGGCCGCATGGACTTGTTCCTATCCACCAGATGAGTTCTGACCTATCA
 |         |         |         |         |         |         |         |         |
 330       340       350       360       370       380       390       400       410

     380       390       400       410       420       430       440       450
     |         |         |         |         |         |         |         |
TTGCTTGCTAACTTCAGTCAACCTGAAATAAACCTACTTACTAATCACACAGAAAATTCTGTCATAAATTTGACCTGCTCAT
 .................................................................................
GTGCTTGCTAACTTCAGTCAACCTGAAATAAACCTACTTACTAATCACACAGAAAATTCTGTCATAAATTTGACCTGCTCAT
        |         |         |         |         |         |         |         |
        420       430       440       450       460       470       480       490

  460       470       480       490       500       510       520       530
  |         |         |         |         |         |         |         |
CTACACAAGGCTACCCAGAACCCCAGAGGATGTATATGTTGCTAAATACGAAGAATTCAACCACTGAGCATGATGCTGACAT
..................................................................................
CTACACAAGGCTACCCAGAACCCCAGAGGATGTATATGTTGCTAAATACGAAGAATTCAACCACTGAGCATGATGCTGACAT
      |         |         |         |         |         |         |         |
      500       510       520       530       540       550       560       570
```

```
540       550       560       570       580       590       600       610       620
 |         |         |         |         |         |         |         |         |
GAAGAAATCTCAAAATAACATCACGGAACTCTACAATGTATCAATCAGGGTGTCTCTTCCCATCCCTCCCGAGACAAATGTG
:::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
GAAGAAATCTCAAAATAACATCACGGAACTCTACAATGTATCAATCAGGGTGTCTCTTCCCATCCCTCCCGAGACAAATGTG
     |         |         |         |         |         |         |         |
    580       590       600       610       620       630       640       650

        630       640       650       660       670       680       690       700
         |         |         |         |         |         |         |         |
AGCATCGTCTGTGTCCTGCAACTTGAGCCAAGCAAGACACTGCTTTTCTCCCTACCTTGTAATATAGATGCAAAGCCACCTG
:::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AGCATCGTCTGTGTCCTGCAACTTGAGCCAAGCAAGACACTGCTTTTCTCCCTACCTTGTAATATAGATGCAAAGCCACCTG
   |         |         |         |         |         |         |         |
  660       670       680       690       700       710       720       730

       710       720       730       740       750       760       770       780
        |         |         |         |         |         |         |         |
TGCAACCCCCTGTCCCAGACCACATCCTCTGGATTGCAGCTCTACTTGTAACAGTGGTCGTTGTGTGTGGGATGGTGTCCTT
:::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
TGCAACCCCCTGTCCCAGACCACATCCTCTGGATTGCAGCTCTACTTGTAACAGTGGTCGTTGTGTGTGGGATGGTGTCCTT
 |         |         |         |         |         |         |         |         |
740       750       760       770       780       790       800       810       820

    790       800       810       820       830       840       850       860
     |         |         |         |         |         |         |         |
TGTAACACTAAGGAAAAGGAAGAAGAAGCAGCCTGGCCCCTCTAATGAATGTGGTGAAACCATCAAAATGAACAGGAAGGCG
:::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
TGTAACACTAAGGAAAAGGAAGAAGAAGCAGCCTGGCCCCTCTAATGAATGTGGTGAAACCATCAAAATGAACAGGAAGGCG
         |         |         |         |         |         |         |         |
        830       840       850       860       870       880       890       900

 870       880       890       900       910       920       930       940
  |         |         |         |         |         |         |         |
AGTGAACAAACTAAGAACAGAGCAGAAGTCCATGAACGATCTGATGATGCCCAGTGTGATGTTAATATTTTAAAGACAGCCT
:::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AGTGAACAAACTAAGAACAGAGCAGAAGTCCATGAACGATCTGATGATGCCCAGTGTGATGTTAATATTTTAAAGACAGCCT
      |         |         |         |         |         |         |         |
     910       920       930       940       950       960       970       980

950       960       970       980       990
 |         |         |         |         |
CAGATGACAACAGTACTACAGATTTTTAATTAAAGAGTAAACTCC
:::::::::::::::::::::::::::::   :     :
CAGATGACAACAGTACTACAGATTTTTAAGTCGACCTCGAGGGGGGGCCCGGTACCAGCTTTTGTT
   |         |         |         |         |         |         |
  990      1000      1010      1020      1030      1040      1050
```

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Contig             ACCATGGGACTGAGTAACATTCTCTTTGTGATGGTCTTCCTGCTCTCT
Murine B7-2        -CCATGGGACTGAGTAACATTCTCTTTGGGATGGTCCTCCTGCTCTCT
Porcine CD68(i)    ACCATGGGCTTGGCAATCCTTATCTTTGTGACAGTCTTGCTGATCTCA
Human B7.2         ACTATGGGACTGAGTAACATTCTCTTTGTGATGGCCTTCCTGCTCTCT

GGTGCTGCTTCCBTGAAGABTCAAGCTTATTTCAATGAGACTGCAGAHCTGCCGTGCCAATTTA
GGTGCTGCCTCCTTGAAAAGTCAGGCATATTTCAATGAGACTGGAGAACTGCCGTGCCATTTTA
GATGCTGTTTCCGTGGAGACGCAAGCTTATTTCAATGGGACTGCATATCTGCCGTGCCCATTTA
GGTGCTGCTCCTCTGAAGATTCAAGCTTATTTCAATGAGACTGCAGACCTGCCATGCCAATTTG

CAAACTCTCAAAACCTAAGCCTGAGTGAGCTGGTAGTATTTTGGCAGGACCAGGAAAACTTGGT
CAAACTCGCAGAACCTAAGCCTGGATGAGCTGGTCATATTTTGGCAGGACCAGGATAACCTGGT
CAAAGGCTCAAAACATAAGCCTGAGTGAGCTGGTAGTATTTTGGCAGGACCAGCAAAAGTTGGT
CAAACTCTCAAAACCAAAGCCTGAGTGAGCTAGTAGTATTTTGGCAGGACCAGGAAAACTTGGT

TCTGTACGAGCTATACTTAGGCAAAGAGAAACTTGATAGTGTTAATTCCAAGTATATGGGCCGC
TCTCTACGAGCTATACCGAGGCCAAGAGAAGCCTCATAATGTTAATTCCAAGTATATGGGTCGC
TCTGTACGAGCACTATTTGGGCACAGAGAAACTTGATAGTGTGAATGCCAAGTACCTGGGCCGC
TCTGAATGAGGTATACTTAGGCAAAGAGAAATTTGACAGTGTTCATTCCAAGTATATGGGCCGC

ACAAGCTTTGACHVGGACAVCTGGACCCTGAGACTTCACAATGTTCAGATCAAGGACAAGGGCT
ACAAGCTTTGACCAGGCCACCTGGACCCTGAGACTCCACAACGTTCAAATCAAGGACAAGGGCT
ACGAGCTTTGACAGGAACAACTGGACTCTACGACTTCACAATGTTCAGATCAAGGACATGGGCT
ACAAGTTTTGATTCGGACAGTTGGACCCTGAGACTTCACAATCTTCAGATCAAGGACAAGGGCT

CGTATCAATGTTTCATCCATCAHAAAVVGCCCACAGGAHTDATTCBCATCCACCAGATGADTTC
CATATCAATGTTTCATCCATCATAAAGGGCCGCATGGACTTGTTCCTATCCACCAGATGAGTTC
CGTATGATTGTTTTATACAAAAAAAGCCACCCACAGGATCAATTATCCTCCAACAGACATTAAC
TGTATCAATGTATCATCCATCACAAAAAGCCCACAGGAATGATTCGCATCCACCAGATGAATTC

TGAACTGTCAGTGCTTGCTAACTTCAGTCAACCTGAAATAAAACTAVTTHCTAATVTAACAGAA
TGACCTATCAGTGCTTGCTAACTTCAGTCAACCTGAAATAAACCTACTTACTAATCACACAGAA
AGAACTGTCAGTGATCGCCAACTTCAGTGAACCTGAAATAAAACTGGCTCAGAATGTAACAGGA
TGAACTGTCAGTGCTTGCTAACTTCAGTCAACCTGAAATAGTACCAATTTCTAATATAACAGAA
```

Figure 5: Comparison of CD86(i) with published sequences for murine and human CD86. Sequence continues overleaf.

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C:  g               AATTCTGDCATAAATTTGACCTGCTCATCTAHACAAGGTTACCCAGAACCTAAGAAGATGTATD
Murine B7-2         AATTCTGTCATAAATTTGACCTGCTCATCTACACAAGGCTACCCAGAACCCCAGAGGATGTATA
Porcine CD68(i)     AATTCTGGCATAAATTTGACCTGCACGTCTAAGCAAGGTCACCCGAAACCTAAGAAGATGTATT
Human B7.2          AATGTGTACATAAATTTGACCTGCTCATCTATACACGGTTACCCAGAACCTAAGAAGATGAGTG

TTTTGCTAAVTACNAAGAATTCAACTAHTGAGTATGATGVTAACATGCAGAAATCTCAAGATAA
TGTTGCTAAATACGAAGAATTCAACCACTGAGCATGATGCTGACATGAAGAAATCTCAAAATAA
TTCTGATAACT------AATTCAACTAATGAGTATGGTGATAACATGCAGATATCACAAGATAA
TTTTGCTAAGAACCAAGAATTCAACTATCGAGTATGATGGTATTATGCAGAAATCTCAAGATAA

TGTCACAGAACTGTACAATGTHTCCATCAGCBTGTCTCTTTCATTCCCTGATGDTACGAGNNAT
CATCACGGAACTCTACAATGTATCAATCAGGGTGTCTCTTCCCATCCCTCCCGAGACAA---AT
TGTCACAGAACTGTTCAGTATCTCCAACAGCCTCTCTCTTTCATTCCCGGATGGTGTGTGGCAT
TGTCACAGAACTGTACGACGTTTCCATCAGCTTGTCTGTTTCATTCCCTGATGTTACGAGCAAT

ATGACCATCGTCTGTGTTCTGGAAACTGAGNCAANCAAGACNCNGCTTTTCTCCHHACCTTTCA
GTGAGCATCGTCTGTGTCCTGCAACTTGAGCCAAGCAAGACACTGCTTTTCTCCCTACCTTGTA
ATGACCGTTGTGTGTGTTCTGGAAACGGAGTCAATGAAGA------TTTCCTCCAAACCTCTCA
ATGACCATCTTCTGTATTCTGGAAACTGA------CAAGACGCGGCTTTTATCTTCACCTTTCT

ATATAGATCHAGAGBHHCCTNNNCAACCTCCTNNCCCAGACCACATBCNNTGGATTACAGCTBT
ATATAGATGCAAAGCCACCTGTGCAACCCCCTGTCCCAGACCACATCCTCTGGATTGCAGCTCT
ATTTCACTCAAGAGTTTCC------ATCTCCTCAAACGTATTGGAAG---GAGATTACAGCTTC
CTATAGAGCTTGAGGACCCT---CAGCCTCC---CCCAGACCACATTCCTTGGATTACAGCTGT

ACTTNNAACAGTGGTCVTTVTVTGTGTGATGGTGTTCTNTVTAATTCTATGGAAANNNAAGAAG
ACTTGTAACAGTGGTCGTTGTGTGTGGGATGGTGTCCTTTGTAACACTAAGGAAA---AGGAAG
AGTT---ACTGTGGCCCTCCTCCTTGTGATGCTGCTC---ATCATTGTATG---TCACAAGAAG
ACTTCCAACAG---TTATTATATGTGTGATGGTTTTCTGTCTAATTCTATGGAAATGGAAGAAG

AAGAAGCAGCCTVGCAVCTCTAATAAATGTGGNNNAACCAHCAAAATGGAGAGGGANGNGAGTG
AAGAAGCAGCCTGGCCCCTCTAATGAATGTGGTGAAACCATCAAAATGAACAGGAAGGCGAGTG
CCGAATCAGCCTAGCAGGCCCAGCAA------CACAGCCTCTAAGTTAGAGCGGGA---TAGT-
AAGAAGCGGCCTCGCAACTCTTATAAATGTGG---AACCAACACAATGGAGAGGGAAGAGAGTG

AACANACTAAGAACAGAGAAAAANTCCATNNACCTGAAVGATCTGATGAAGCCCAGNGTGNTNT
AACAAACTAAGAACAGAGCAGAAGTCCAT------GAACGATCTGATGATGCCCAGTGTGATGT
AACG--CTG---ACAGAGAGA----CTATCAACCTGAAGGAACT--TGAACCCCA---------
AACAGACCAAGAAAAGAGAAAAAATCCATATACCTGAAAGATCTGATGAAGCCCAGCGTGTTTT

TAANADTTNNAAGACAGCTTCANNNGACAAAAGTNNTACANNTTTTTAADTNNAGAGTNAAGNN
TAATATTTTAAAGACAGCCTCAGATGACAACAGTACTACAGATTTTTAAGT-------------
----AATT--------GCTTCA----GCAAAA-------------CCAAATGCAGAGTGAAG--
TAAAAGTTCGAAGACATCTTCATGCGACAAAAGTGATACATGTTTTTAATTAAAGAGTAAAGCC
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                          10        20        30        40        50
                           |         |         |         |         |
Contig                        ...| ||.   |.|.|. ||  ..... .| ... .|||..
Murine CD86        MDPRC-------TMGLAILIFVTVLLISDAVSVETQAYFNGTAYLPCPFTKAQNI
Porcine CD86(i)    --PRCGRSRTSGSMGLSNILFGMVLLLSGAASLKSQAYFNETGELPCHFTNSQNL
Human CD86         -------------MGLSNILFVMAFLLSGAAPLKIQAYFNETADLPCQFANSQNQ
Porcine CD86       -------------MGLSNILFVMVLLLSGAASLKSQAYFNETGELPCHFTNSQNL

       60        70        80        90       100       110
        |         |         |         |         |         |
.. ...|.....||... . . . .. ||.||..|......  .......|...
SLSELVVFWQDQQKLVLYEHYLGTEKLDSVNAKYLGRTSFDRNNWTLRLHNVQIK
SLDELVIFWQDQDNLVLYELYRGQEKPHNVNSKYMGRTSFDQATWTLRLHNVQIK
SLSELVVFWQDQENLVLNEVYLGKEKFDSVHSKYMGRTSFDSDSWTLRLHNLQIK
SLDELVIFWQDQDNLVLYELYRGQEKPHNVNSKYMGRTSFDQATWTLRLHNVQIK

        120       130       140       150       160
          |         |         |         |         |
. . .|.|.| . . . | ||. ||..||....|...    . . .  .....|
DMGSYDCFIQKKPPTGSIILQQTLTELSVIANFSEPEIKLAQNVTGNSGINLTCT
DKGSYQCFIHHKGPHGLVPIHQMSSDLSVLANFSQPEINLLTNHTENSVINLTCS
DKGLYQCIIHHKKPTGMIRIHQMNSELSVLANFSQPEIVPISNITENVYINLTCS
DKGSYQCFIHHKGPHGLVPIHQMSSDLSLLANFSQPEINLLTNHTENSVINLTCS

    170       180       190       200       210       220
      |         |         |         |         |         |
. |. . .||. .|  ... . | .| ..|.|...|||.  |.|||.   |
SKQGHPKPKKMYFLIT--NSTNEYGDNMQISQDNVTELFSISNSLSLSFPDGVWH
STQGYPEPQRMYMLLNTKNSTTEHDADMKKSQNNITELYNVSIRVSLPIPPET-N
SIHGYPEPKKMSVLLRTKNSTIEYDGIMQKSQDNVTELYDVSISLSVSFPDVTSN
STQGYPEPQRMYMLLNTKNSTTEHDADMKKSQNNITELYNVSIRVSLPIPPET-N

         230       240       250       260       270
           |         |         |         |         |
|||.|  .  .   |     |   .      |  .   |  |.|  |.
MTVVCVLETESMKISSKPLNFTQEFPSP-------QTYW-KEITASVTVALLLVM
VSIVCVLQLEPSKTLLFSLPCNIDAKPPVQPPVPDHILWIAALLVTVVVVCGMVS
MTIFCI--LETDKTRLLSSPFSIELEDP-QPP-PDHIPWITAVLPTVII-CVMVF
VSIVCVLQLEPSKTLLFSLPCNIDAKPPVQPPVPDHILWIAALLVTVVVVCGMVS

   280       290       300       310       320       330
     |         |         |         |         |         |
| |  |. ||. |     | ||. | |  . ||      | |
LLIIVCHKKPNQPSRPSN--TASKLERDSNAD---RETINL----KELEPQIASA
FVTLRK-RKKKQPGPSNECGETIKMNRKASEQTKNRAEVH--ERSDDAQCDVNIL
CLILWKWKKKKRPRNSYKCG-TNTMEREESEQTKKREKIHIPERSDEAQRVFKSS
FVTLRK-RKKKQPGPSNECGETIKMNRKASEQTKNRAEVH--ERSDDAQCDVNIL

       340       350
         |         |
. |.|. .     .|  .....
KPNAE
KTASDDNSTTDFXVDLEGGPGTSFC
KTSSCDKSDTCF
KTASDDNSTT--DFXLKSKL
```

Figure 6: Predicted amino acid sequence for CD86(i) compared with those for pig, human and mice.

Figure 7: Position of 5' and 3' internal and external porcine B7-1 primers with respect to human and murine B7-1 nucleotide sequences. Primer sequences are underlined and labelled as follows. Internal primers (A) and external primers (B).

```
        10        20        30        40        50        60        70        80
CCAAAGAAAAAGTGATTTGTCATTGCTTTATAGACTGTAAGAAGAGAACATCTCAGAAGTGGAGTCTTACCCTGAAATCAAA
                                                            GAGTTTTATACCTCAATAGACT
                                                                     10         20

      90       100       110       120       130       140       150       160
GGATTTAAAGAAAAAGTGGAATTTTTCTTCAGCAAGCTGTGAAACTAAATCCACAACCTTTGGAGACCCAGGAACACCCTCC
CTTACTAGTTTCTCTTTTTCAGGTTGTGAAACTCAACCTTCAAAGACACTCTGTTCCATTTCTGTGGACTAATAGGATCATC
      30        40        50        60        70        80        90       100

      170       180       190       200       210       220       230       240
AATCTCTGTGTGTTTTGTAAACATCACTGGAGGGTCTTCTACGTGAGCAATTGGATTGTCATCAGCCCTGCCTGTTTTGCAC
TTTAGCATCTGCCGGGTGGATGCCATCCAGGCTTCTTTTTCTACATCTCTGTTTCTCGATTTTTGTGAGCCTAGGAGGTGCC
      110       120       130       140       150       160       170       180

   250       260       270       280       290       300       310       320
CTGGGAAGTGCCCTGGTCTTACTTGGGTCCAAATTGTTGGCTTTCACTTTTGACCCTAAGCATCTGAAGCCATGGGCCACAC
TAAGCTCCATTGGCTCTAGATTCCTGGCTTTCCCCATCATGTTCTCCAAAGCATCTGAAGCTATGGCTTGCAATTGTCAGTT
   190       200       210       220       230       240       250       260

330       340       350       360       370       380       390       400       410
ACGGAGGCAGGGAACATCACCATCCAAGTGTCCATACCTCAATTTCTTTCAGCTCTTGGTGCTGGCTGGTCTTTCTCACTTC
GATGCAGGATACACCACTCCTCAAGTTTCCATGTCCAAGGCTCATTCTTCTCTTTGTGCTGCTGATTCGTCTTTCACAAGTG
270       280       290       300       310       320       330       340       350

      420       430       440       450       460       470       480       490
TGTTCAGGTGTTATCCACGTGACCAAGGAAGTGAAAGAAGTGGCAACGCTGTCCTGTGGTCACAATGTTTCTGTTGAAGAGC
TCTTCAGATGTTGATGAACAACTGTCCAAGTCAGTGAAAGATAAGGTATTGCTGCCTTGCCGTTACAACTCTCCTCATGAAG
      360       370       380       390       400       410       420       430

     500       510       520       530       540       550       560       570
TGGCACAAACTCGCATCTACTGGCAAAAGGAGAAGAAAATGGTGCTGACTATGATGTCTGGGGACATGAATATATGGCCCGA
ATGAGTCTGAAGACCGAATCTACTGGCAAAAACATGACAAAGTGGTGCTGTCTGTCATTGCTGGGAAACTAAAAGTGTGGCC
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     580       590       600       610       620       630       640       650
TACAAGAACCGGACCATCTTTGATATCACTAATAACCTCTCCATTGTGATCCTGGCTCTGCGCCCATCTGACGAGGGCACA
CGAGTATAAGAACCGGACTTTATATGACAACACTACCTACTCTCTTATCATCCTGGGCCTGGTCCTTTCAGACCGGGGCACA
     520       530       540       550       560       570       580       590

     660       670       680       690       700       710       720       730
TACGAGTGTGTTGTTCTGAAGTATGAAAAAGACGCTTTCAAGCGGGAACACCTGGCTGAAGTGACGTTATCAGTCAAAGCTG
TACAGCTGTGTCGTTCAAAAGAAGGAAAGAGGAACGTATGAAGTTAAACACTTGGCTTTAGTAAAGTTGTCCATCAAAGCTG
     600       610       620       630       640       650       660       670

 740       750       760       770       780       790       800       810       820
ACTTCCCTACACCTAGTATATCTGACTTTGAAATTCCAACTTCTAATATTAGAAGGATAATTTGCTCAACCTCTGGAGGTTT
ACTTCTCTACCCCCAACATAACTGAGTCTGGAAACCCATCTGCAGACACTAAAAGGATTACCTGCTTTGCTTCCGGGGGTTT
 680       690       700       710       720       730       740       750       760

      830       840       850       860       870       880       890       900
TCCAGAGCCTCACCTCTCCTGGTTGGAAAATGGAGAAGAATTAAATGCCATCAACACAACAGTTTCCCAAGATCCTGAAACT
CCCAAAGCCTCGCTTCTCTTGGTTGGAAAATGGAAGAGAATTACCTGGCATCAATACGACAATTTCCCAGGATCCTGAATCT
      770       780       790       800       810       820       830       840

     910       920       930       940       950       960       970       980
AGCTCTATGCTGTTAGCAGCAAACTGGATTTCAATATGACAACCAACCACAGCTTCATGTGTCTCATCAAGTATGGACATT
GAATTGTACACCATTAGTAGCCAACTAGATTTCAATACGACTCGCAACCACACCATTAAGTGTCTCATTAAATATGGAGATG
     850       860       870       880       890       900       910       920

     990       1000      1010      1020      1030      1040      1050      1060
TAAGAGTGAATCAGACCTTCAACTGGAATACAACCAAGCAAGAGCATTTTCCTGATAACCTGCTCCCATCCTGGGCCATTAC
CTCACGTGTCAGAGGACTTCACCTGGGAAAAACCCCCAGAAGACCCTCCTGATAGCAAGAACACACTTGTGCTCTTTGGGGC
     930       940       950       960       970       980       990       1000

   1070      1080      1090      1100      1110      1120      1130      1140
CTTAATCTCAGTAAATGGAATTTTTGTGATATGCTGCCTGACCTACTGCTTTGCCCCAAGATGCAGAGAGAGAAGGAGGAAT
AGGATTCGGCGCAGTAATAACAGTCGTCGTCATCGTTGTCATCATCAAATGCTTCTGTAAGCACAGAAGCTGTTTCAGAAGA
   1010      1020      1030      1040      1050      1060      1070      1080
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1150      1160      1170      1180      1190      1200      1210      1220      1230
CAGAGATTGAGAAGGAAAGTGTACGCCCTGTATAACAGTGTCCGCAGAAGCAAGGGGCTGAAAAGATCTGAAGGTAGCCTC
AATGAGGCAAGCAGAGAAACAAACAACAGCCTTACCTTCGGGCCTGAAGAAGCATTAGCTGAACAGACCGTCTTCCTTTAGT
1090      1100      1110      1120      1130      1140      1150      1160      1170

     1240      1250      1260      1270      1280      1290      1300      1310
CGTCATCTCTTCTGGGATACATGGATCGTGGGATCATGAGGCATTCTTCCCTTAACAAATTTAAGCTGTTTTACCCACTAC
TCTTCTCTGTCCATGTGGGATACATGGTATTATGTGGCTCATGAGGTACAATCTTTCTTTCAGCACCGTGCTAGCTGATCTT
     1180      1190      1200      1210      1220      1230      1240      1250

     1320      1330      1340      1350      1360      1370      1380      1390
CACCTTCTTAAAAACCTCTTTCAGATTAAGCTGAACAGTTACAAGATGGCTGGCATCCCTCTCCTTTCTCCCCATATGCA
TCGGACAACTTGACACAAGATAGAGTTAACTGGAAGAGAAAGCCTTGAATGAGGATTTCTTTCCATCAGGAAGCTACGGGC
     1260      1270      1280      1290      1300      1310      1320      1330

     1400      1410      1420      1430      1440      1450      1460      1470
ATTTGCTTAATGTAACCTCTTCTTTTGCCATGTTTCCATTCTGCCATCTTGAATTGTCTTGTCAGCCAATTCATTATCTATT
AAGTTTGCTGGGCCTTTGATTGCTTGATGACTGAAGTGGAAAGGCTGAGCCCACTGTGGGTGGTGCTAGCCCTGGGCAGGGG
     1340      1350      1360      1370      1380      1390      1400      1410

   1480      1490
CACTAATTTGAG
CAGGTGACCCTGGGTGGTATAAGAAAAAGAGCTGTCACTAAAAGGAGAGGTGCCTAGTCTTACTGCAACTTGATATGTCATG
   1420      1430      1440      1450      1460      1470      1480      1490

TTTGGTTGGTGTCTGTGGGAGGCCTGCCCTTTTCTGAAGAGAAGTGGTGGAGAGTGGATGGGGTGGGGGCAGAGGAAAAGT
 1500      1510      1520      1530      1540      1550      1560      1570      1580

GGGGGAGAGGGCCTGGAGGAGAGGAGGGAGGGGGACGGGGTGGGGGTGGGAAAACTATGGTTGGGATGTAAAAACGGATA
        1590      1600      1610      1620      1630      1640      1650      1660
```

Figure 8A: CD40 nucleotide sequence comparison between human, murine and cattle sequences.

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                  10        20        30        40        50        60
Contig      NNNNNNNNNNNNNNNNNNNNNNNNNTGCCNNCTGNNNNNNNCTCGCCATGGTTCGTTTGCCTCTGCAG
Human CD40  GCCTCGCTCGGGCGCCCAGTGGTCCTGCCGCCTGGTCTCACCTCGCCATGGTTCGTCTGCCTCTGCAG
Bovine CD40 -----------------------------------------CTCGCCATGGTTCGTTTGCCACTGCAG
Mouse CD40  -------------------------TGCC--CTG------------CATGGTGTCTTTGCCTCGGCTG

            70        80        90        100       110       120       130
Contig      TGCGTCCTCTGGGGCTGCTTGCTGACCGCBGTCCATCCAGAACCABCCACTGCDTGCAGAGAVAAACA
Human CD40  TGCGTCCTCTGGGGCTGCTTGCTGACCGCTGTCCATCCAGAACCACCCACTGCATGCAGAGAAAAACA
Bovine CD40 TGTCTCTTCTGGGGCTTCTTTCTGACCGCCGTCCACTCAGAACCAGCCACTGCTTGTGGAGAGAAGCA
Mouse CD40  TGCGCGCTATGGGGCTGCTTGTTGACAGCGGTCCATCTAGGGCAGTGTGTTACGTGCAGTGACAAACA

              140       150       160       170       180       190       200
Contig      GTACCTAVTVAACAGTCAGTGCTGTGATTTGTGCCAGCCAGGACAGAAACTGGTGAGCGACTGCACAG
Human CD40  GTACCTAATAAACAGTCAGTGCTGTTCTTTGTGCCAGCCAGGACAGAAACTGGTGAGTGACTGCACAG
Bovine CD40 ATACCCAGTGAACAGTCTTTGCTGTGATTTGTGCCCGCCGGGACAGAAACTGGTGAACGACTGCACAG
Mouse CD40  GTACCTCCACGATGGCCAGTGCTGTGATTTGTGCCAGCCAGGAAGCCGACTGACAAGCCACTGCACAG

                210       220       230       240       250       260       270
Contig      AGBTCAVBAAAACVGAATGCCABCCHTGCGGTDAAGGCGAATTCTTAGCCACCTGGAACAGAGAGAHA
Human CD40  AGTTCACTGAAACGGAATGCCTTCCTTGCGGTGAAAGCGAATTCCTAGACACCTGGAACAGAGAGACA
Bovine CD40 AGGTCAGCAAAACAGAATGCCAGTCCTGCGGTAAAGGCGAATTCTTGTCCACCTGGAACAGAGAGAAA
Mouse CD40  CTCTTGAGAAGACCCAATGCCACCCATGTGACTCAGGCGAATTCTCAGCCCAGTGGAACAGGGAGATT

                  280       290       300       310       320       330       340
Contig      CACTGTCACCAGCACAGATACTGCGACCCCAACCTAGGGCTTCGGGTCCAGAAGGAGGGCACCTCAGA
Human CD40  CACTGCCACCAGCACAAATACTGCGACCCCAACCTAGGGCTTCGGGTCCAGCAGAAGGGCACCTCAGA
Bovine CD40 TACTGTCACGAGCACAGATACTGCAACCCCAACCTAGGGCTCCGGATCCAGAGCGAGGGTACCTTGAA
Mouse CD40  CGCTGTCACCAGCACAGACACTGTGAACCCAATCAAGGGCTTCGGGTTAAGAAGGAGGGCACCGCAGA

                    350       360       370       380       390       400
Contig      AACAGACACCATCTGTACCTGTGAVGAAGGCCAACACTGTACCAGTVAGGCCTGCGAGAGHTGTGCBC
Human CD40  AACAGACACCATCTGCACCTGTGAAGAAGGCTGGCACTGTACGAGTGAGGCCTGTGAGAGCTGTGTCC
Bovine CD40 TACAGACACCATTTGTGTATGTGTCGAAGGCCAACACTGTACCAGTCACACCTGCGAAAGTTGCACGC
Mouse CD40  ATCAGACACTGTCTGTACCTGTAAGGAAGGACAACACTGCACCAGCAAGGATTGCGAGGCATGTGCTC

            410       420       430       440       450       460       470
Contig      HGCACAGCTCVTGTHTCCCTGGCTTTGGGGTCAAGCAGATBGCTACAGGGVTTTCTGATACCGTCTGT
Human CD40  TGCACCGCTCATGCTCGCCCGGCTTTGGGGTCAAGCAGATTGCTACAGGGGTTTCTGATACCATCTGC
Bovine CD40 CCCACAGCTTGTGTCTCCCTGGCTTCGGGGTCAAGCAGATCGCTACAGGGCTTTTGGATACCGTCTGT
Mouse CD40  AGCACACGCCCTGTATCCCTGGCTTTGGAGTTATGGAGATGGCCACTGAGACCACTGATACCGTCTGT

              480       490       500       510       520       530       540
Contig      GADCCCTGCCCAGTCGGCTTCTTCTCCAATGTGTCATCTGCTTTCGAAAAGTGTCACCCTTGGACAAG
Human CD40  GAGCCCTGCCCAGTCGGCTTCTTCTCCAATGTGTCATCTGCTTTCGAAAAATGTCACCCTTGGACAAG
Bovine CD40 GAACCCTGCCCGCTCGGCTTCTTCTCCAACGTGTCATCTGCTTTTGAAAAGTGTCACCGTTGGACAAG
Mouse CD40  CATCCCTGCCCAGTCGGCTTCTTCTCCAATCAGTCATCACTTTCGAAAAGTGTTATCCCTGGACAAG

                550       560       570       580       590       600       610
Contig      CTGTGAGAVHAAAGACCTGGTGGTVCAACAGGHAGGVACGAACAAGACTGATGTTGTCTGTGGTTTCC
Human CD40  CTGTGAGACCAAAGACCTGGTTGTGCAACAGGCAGGCACAAACAAGACTGATGTTGTCTGTGGTCCCC
Bovine CD40 CTGCGAGAGAAAAGGCCTGGTGGAACAACACGTGGGGACGAACAAGACAGATGTTGTCTGCGGTTTCC
Mouse CD40  CTGTGAGGATAAGAACTTGGAGGTCCTACAGAAAGGAACGAGTCAGACTAATGTCATCTGTGGTTTAA
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Contig        AGDVTCGGATGAGAGCCCTGGTGGTGATCCCCGTCATGATGGGVATCCTGTTTGCCATCCTCTTGGTG
Human CD40    AGGATCGGCTGAGAGCCCTGGTGGTGATCCCCATCATCTTCGGGATCCTGTTTGCCATCCTCTTGGTG
Bovine CD40   AGAGTCGGATGAGGACCCTGGTGGTGATCCCCGTCACGATGGGAGTCTTGTTTGCTGTCCTGTTGGTA
Mouse CD40    AGTCCCGGATGCGAGCCCTGCTGGTCATTCCTGTCGTGATGGGCATCCTCATCACCATTTTCGGGGTG

                    690       700       710       720       730       740
                     |         |         |         |         |         |
Contig        TTTGTCTDTATCAAAAAGGTGGCCAAGAAGCCAACVGATAANNNGGCCCTVCACCCTANGGCTNNANG
Human CD40    CTGGTCTTTATCAAAAAGGTGGCCAAGAAGCCAACCAATAA---GGCCCCCACCCCA---------A
Bovine CD40   TCTGCCTGTATCAGGAACATAACCAAGAAGC-GGCAGCTAA---GGCCCTGCACCCTATGGCTGAAAG
Mouse CD40    TTTCTCTATATCAAAAAGGTGGTCAAGAAACCAAAGGATAATGAGATGTTACCCCCTGCGGCTCGACG

              750       760       770       780       790       800       810
               |         |         |         |         |         |         |
Contig        GCAGGATCCCCAGGAGATGANTNNTCCNGAVGATTTTCCCGGCCCCAACACCGCTGCTCCAGTGCAGG
Human CD40    GCAGGAACCCCAGGAGATCAATTTTCCCGACGATCTTCCTGGCTCCAACACTGCTGCTCCAGTGCAGG
Bovine CD40   GCAGGATCCCGTGGAGACGATTGATCCGGAGGATTTTCCCGGCCCCCAC-CCGCCTCTCCGGTGCAAG
Mouse CD40    GCAAGATCCCCAGGAGATG---------GAAGATTATCCCGGTCATAACACCGCTGCTCCAGTGCAGG

                820       830       840       850       860       870       880
                 |         |         |         |         |         |         |
Contig        AGACHTTACACGGGTGTCAGCCGGTCACCCAGGAGGATGGCAAAGAGAGTCGCATCTCAGTGCAGGAG
Human CD40    AGACTTTACATGGATGCCAACCGGTCACCCAGGAGGATGGCAAAGAGAGTCGCATCTCAGTGCAGGAG
Bovine CD40   AGACCTTATGCTGGTGTCAGCCGGTCGCCCAGGAGGACGGCAAAG
Mouse CD40    AGACACTGCACGGGTGTCAGCCTGTCACACAGGAGGATGGTAAAGAGAGTCGCATCTCAGTGCAGGAG

                 890       900       910       920       930       940       950
                  |         |         |         |         |         |         |
Contig        CGGCAGGTGACAGACAGCATAGCCTTGAGGCCCCTGGTCTGMACCCTGGAACYGCTTYRGRRGYGATG
Human CD40    -----------AGACAG--------TGAGGC--------TGCACCC----ACC----CAGGAGTG-TG
Mouse CD40    CGGCAGGTGACAGACAGCATAGCCTTGAGGCCCCTGGTCTGAACCCTGGAACTGCTTTGGAGGCGATG

                  960       970       980       990      1000      1010      1020
                   |         |         |         |         |         |         |
Contig# 1     GCYRCTTGCTGACCTTTGAAGTTTGAGRTGRGCCAARACAGAGCCCAGTGCAGYTRRCYCTCATGCCT
Human CD40    GCCAC-----------------------GTGGGC--AAACAG--------GCAGTTGGCC---------
Mouse CD40    GCTGCTTGCTGACCTTTGAAGTTTGAGATGAGCCAAGACAGAGCCCAGTGCAGCTAACTCTCATGCCT
```

```
                              10        20        30        40        50        60
                               |         |         |         |         |         |
Contig                 •• ••  • |•• |•••••     |• |•••  || •• •• •• |• ||••     •|•
bovine CD40 protein    MVRLPLQCLFWGFFLTAVHSEPATACGEKQYPVNSLCCDLCPPGQKLVNDCTEVSKTECQ
human CD40 protein     MVRLPLQCVLWGCLLTAVHPEPPTACREKQYLINSQCCSLCQPGQKLVSDCTEFTETECL
murine CD40 protein    MVSLPRLCALWGCLLTAVHLGQCVTCSDKQYLHDGQCCDLCQPGSRLTSHCTALEKTQCH

                              70        80        90       100       110       120
                               |         |         |         |         |         |
Contig                 |•| |••   ••••  ••|•| •|•• •••||  •• ||••|• • •• ••••  ••|•
bovine CD40 protein    SCGKGEFLSTWNREKYCHEHRYCNPNLGLRIQSEGTLNTDTICVCVEGQHCTSHTCESCT
human CD40 protein     PCGESEFLDTWNRETHCHQHKYCDPNLGLRVQQKGTSETDTICTCEEGWHCTSEACESCV
murine CD40 protein    PCDSGEFSAQWNREIRCHQHRHCEPNQGLRVKKEGTAESDTVCTCKEGQHCTSKDCEACA

                             130       140       150       160       170       180
                               |         |         |         |         |         |
Contig                  •  • •••••  ||••   ••|•|•••|••••• •• ••••  ••••• •|•   | ••|
bovine CD40 protein    PHSLCLPGFGVKQIATGLLDTVCEPCPLGFFSNVSSAFEKCHRWTSCERKGLVEQHVGTN
human CD40 protein     LHRSCSPGFGVKQIATGVSDTICEPCPVGFFSNVSSAFEKCHPWTSCETKDLVVQQAGTN
murine CD40 protein    QHTPCIPGFGVMEMATETTDTVCHPCPVGFFSNQSSLFEKCYPWTSCEDKNLEVLQKGTS

                             190       200       210       220       230       240
                               |         |         |         |         |         |
Contig                 |•|•|•• | •|•|•|•••|  •|•|||| •   •||| ••            |  •  |
bovine CD40 protein    KTDVVCGFQSRMRTLVVIPVTMGVLFAVLLVSACIRNITKK------------RQLRPCTL
human CD40 protein     KTDVVCGPQDRLRALVVIPIIFGILFAILLVLVFIKKVAKKPTNKAPHP----KQEPQEI
murine CD40 protein    QTNVICGLKSRMRALLVIPVVMGILITIFGVFLYIKKVVKKPKDNEMLPPAARRQDPQEM

                             250       260       270       280
                               |         |         |         |
Contig                    |  •       ||  | |  •    ||||  |    •     ••••••••
bovine CD40 protein    WLKGRIPWRRL---IRRIFPA--PTRLSGARDLMLVSAGRPGGRQ
human CD40 protein     NFPDDLPGSNTAAPVQETLHGCQPVTQEDGKESRISVQERQ
murine CD40 protein    ---EDYPGHNTAAPVQETLHGCQPVTQEDGKESRISVQERQVTDSIALRPLV
```

Figure 8B: Amino acid comparison between human, murine and cattle CD40 sequences.

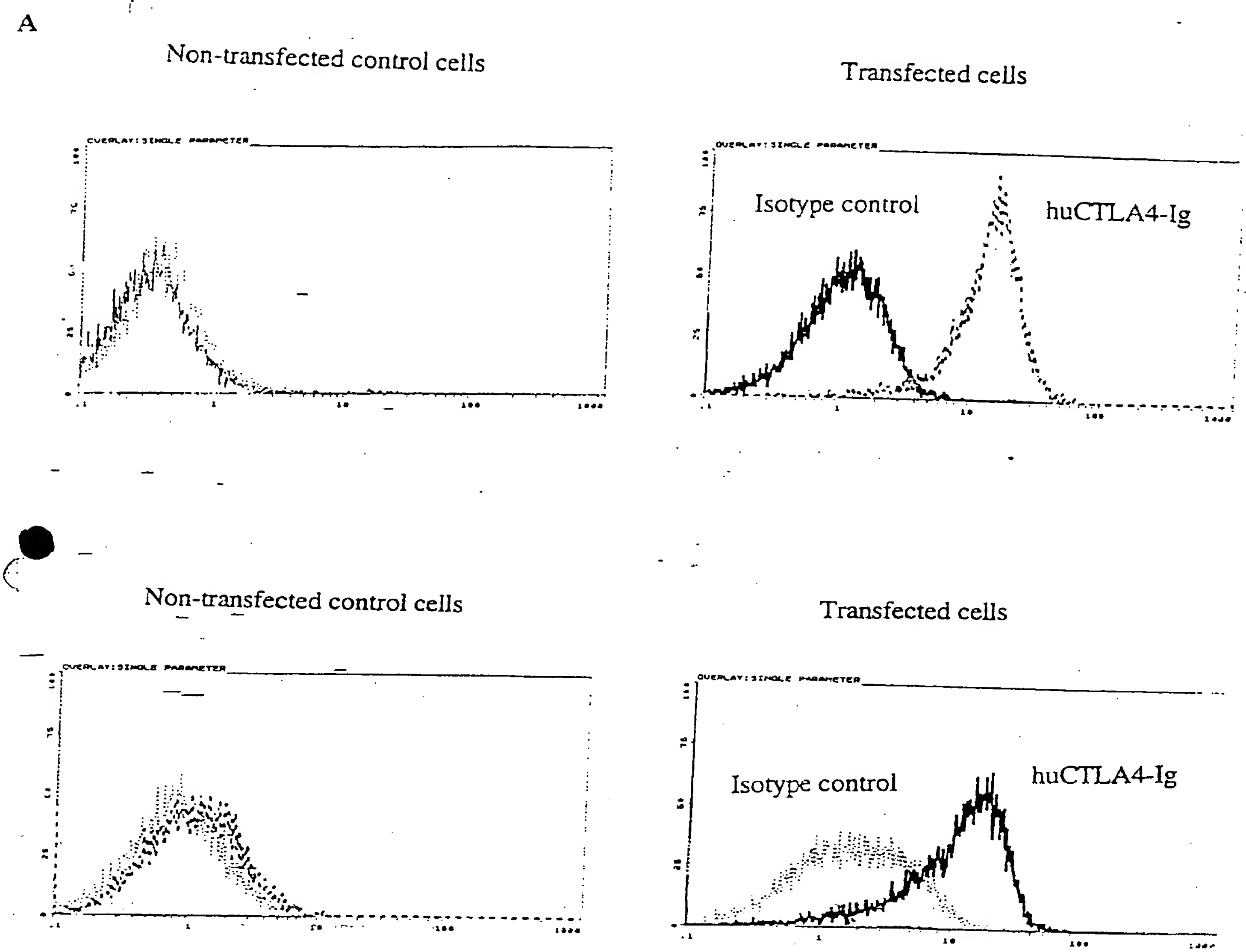


Figure 9: M1-poB7-2 (A) and P815-poB7-2 (B) clones generated by calcium phosphate transfection followed by dynabead selection and cloning by limiting dilution. Expression of B7-2 on the surface of transfected or control cells as determined by fluorescence activated cell sorting. 2.5×10^5 cells were stained with Mab to B7-2 (huCTLA4Ig) or isotype control (huIg) at 1 g/ml. After washing, cells were incubated with goat anti-mouse Ig-FITC conjugate, fixed with 1% paraformaldehyde and analysed on a Coulter counter.

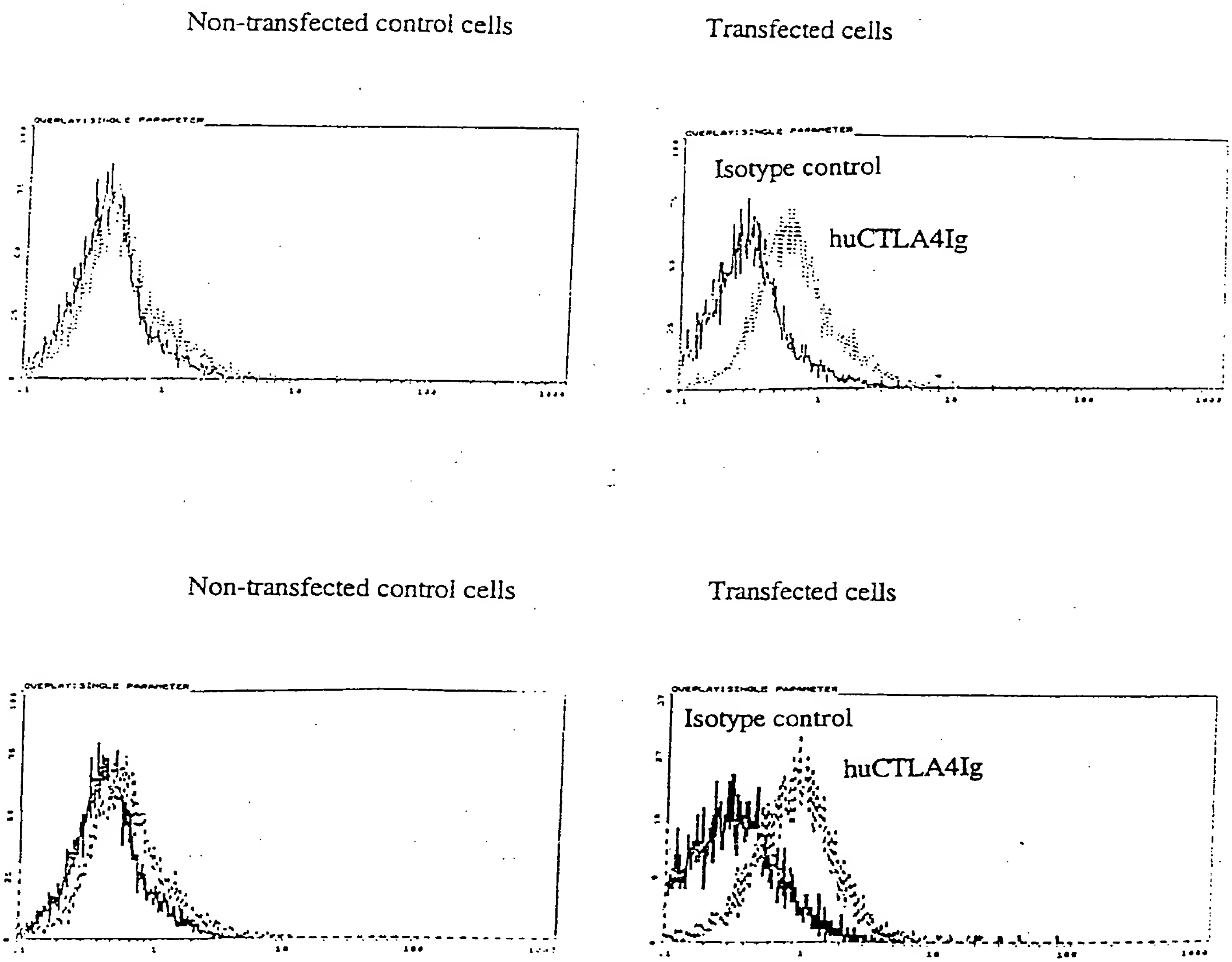
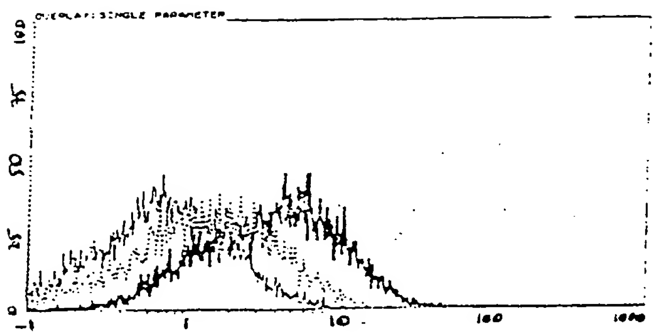


Figure 10: Transient transfections of M1 (A) and P815 (B) cells with CD86(i) by calcium phosphate precipitation .Surface expression of B7-2 on transfected or control cells was determined by fluorescence activated cell sorting.48 hours after transfection, 2.5×10^5 cells were stained with Mab to B7-2 (huCTLA4Ig) or isotype control (huIg) at 1 g/ml. After washing, cells were incubated with goat anti-mouse Ig-FITC conjugate, fixed with 1% paraformaldehyde and analysed on a Coulter counter..

A



B

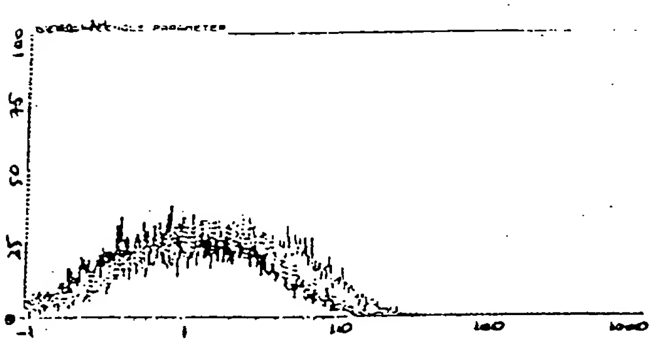


Figure 11 Flow cytometric analysis of porcine B7-2 transfected P815 cells following staining with porcine B7-2-specific sera or ovalbumin peptide control sera. 2.5×10^5 P815 cells were stained with 1/100 of each sera from B7-2 peptide (A) or ova control peptide (B) immunised mice. After washing, cells were incubated with goat anti-mouse IgG (H & L)-HRP and subsequently, Streptavidin-FITC. Cells were fixed with 1% paraformaldehyde and analysed on a Coulter counter.

1 MGLSNILFVM VLLLSGAASL KSQAYFNETG ELPCHFTNSQ

41 NLSLDELVIF WQDQDNLVLY ELYRGQEKPH NVNSKYMGRT

81 SFDQATWTLR LHNVQIKDKG SYQCFIHHKG PHGLVPIHQM

121 SSDLSLLANF SQPEINLLTN HTENSVINLT CSSTQGYPEP

161 QRMYMLLNTK NSTTEHDADM KKSQNNITEL YNVSIRVSLP

201 IPPETNVSIV CVLQLEPSKT LLFSLPCNID AKPPVQPPVP

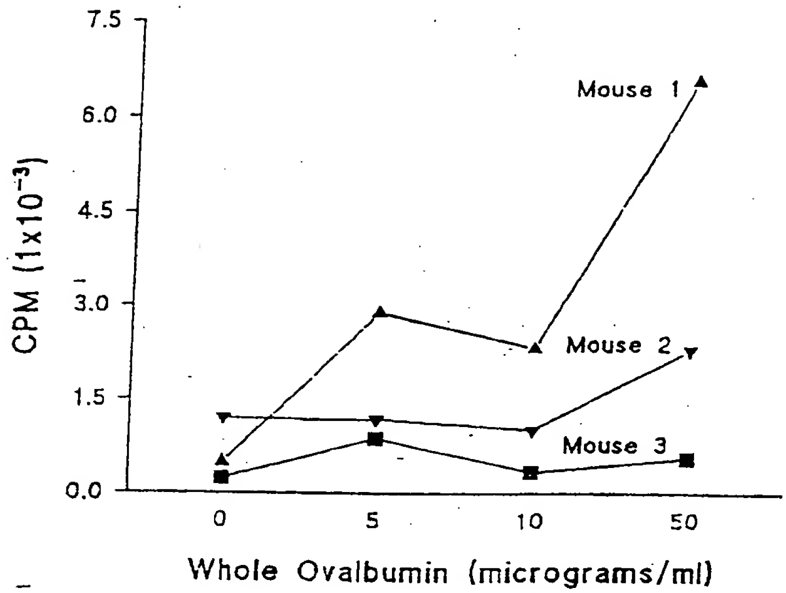
241 DHILWIAALL VTVVVVCGMV SFVTLRKRKK KQPGPSNECG

281 ETIKMNRKAS EQTKNRAEVH ERSDDAQCDV NILKTASDDN

321 STTDF•LKSK L

Figure 12: Positions of the nine B7-2 peptides with respect to the predicted amino acid sequence of porcine B7-2

A



B

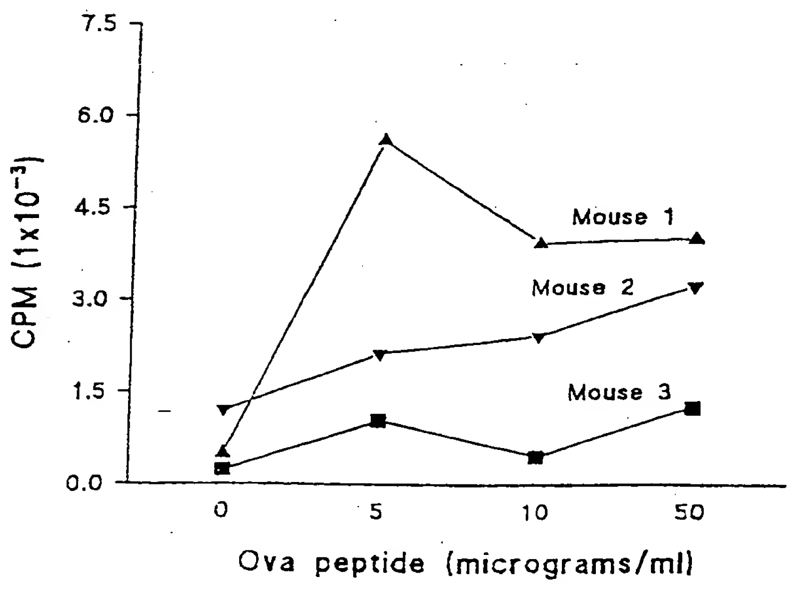
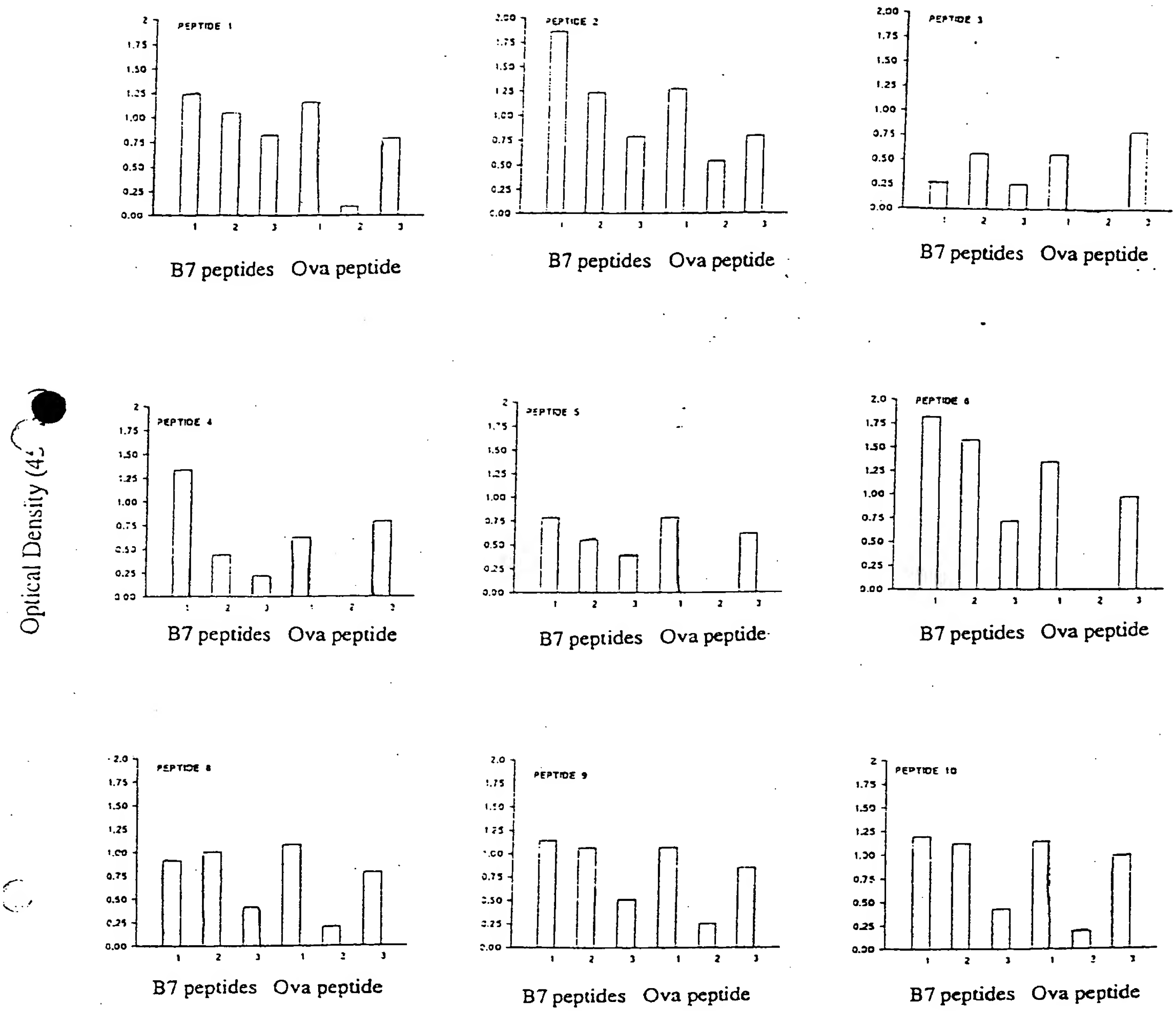


Figure 13: Comparison of *in vitro* T cell proliferation response to whole ovalbumin (A) or $Ova_{323\text{-}339}$ peptide (B). 2.5 x 10^5 T cells and 2.5 x 10^5 APC were plated per well with the indicated concentrations of whole ovalbumin or ova peptide. Cells were cultured for 72 hours in a total volume of 200 1 10% RPMI. T cell proliferation was assayed by the incorporation of ^{3}H-thymidine.



1:300 dilution of sera from immunised mice

Figure 14a Differential binding of B7-2 specific peptide sera or ova control sera as determined by Peptide ELISA. 96 well plates were pre-coated with the nine individual B7-2 specific peptides (P1-6 ; P8-10). Sera harvested from 3 individual B7-2 peptide (Bars 1-3), or 3 individual Ova control peptide (Bars 4-6) immunised mice were then screened for binding. Sera were detected by subsequent incubations with goat anti-mouse IgG-Biotin, Streptavidin-HRP and then developed with TMB. Plates were read at 450nm. Values have been adjusted for binding to no-peptide control plate and represent means for duplicate wells.

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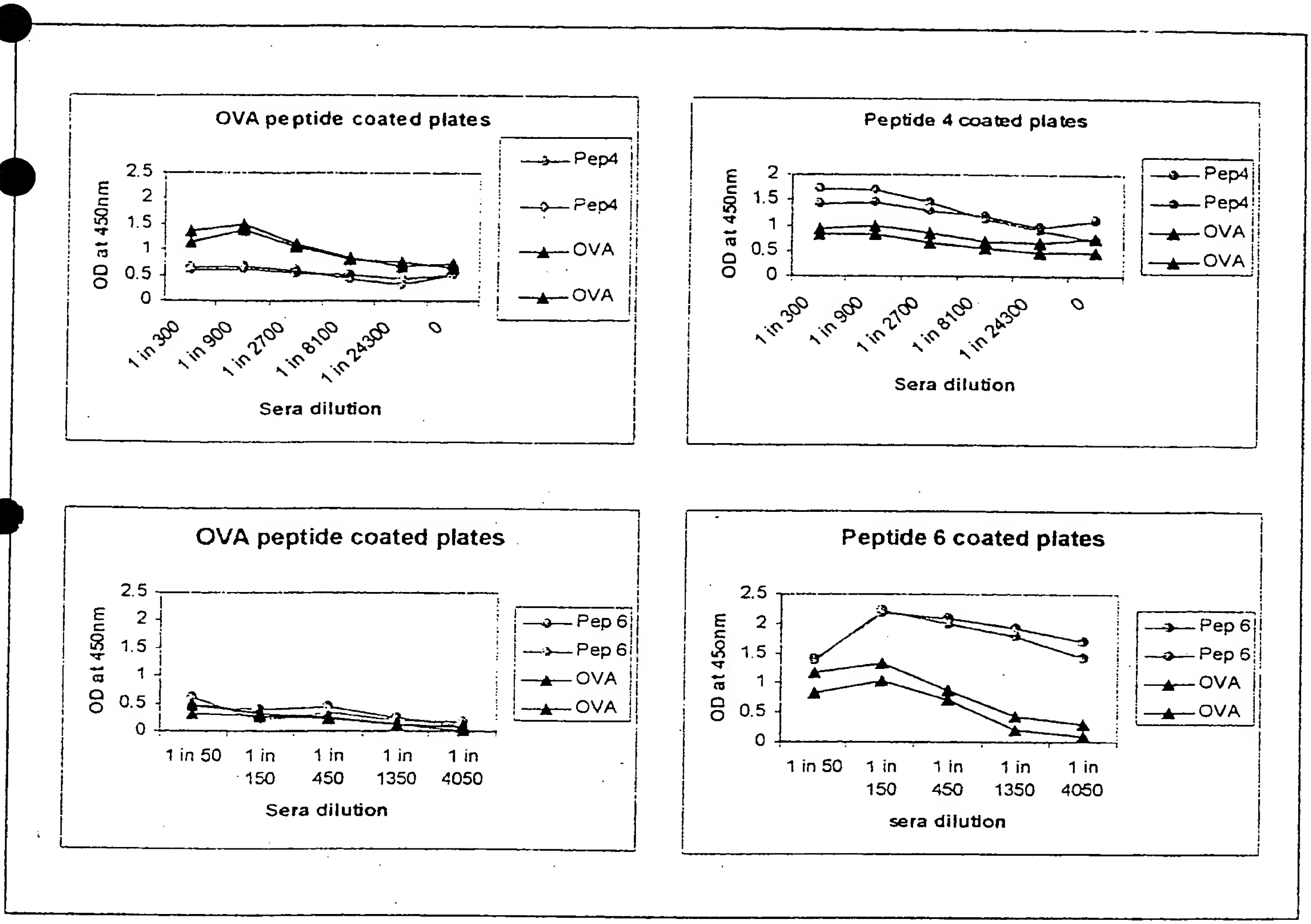
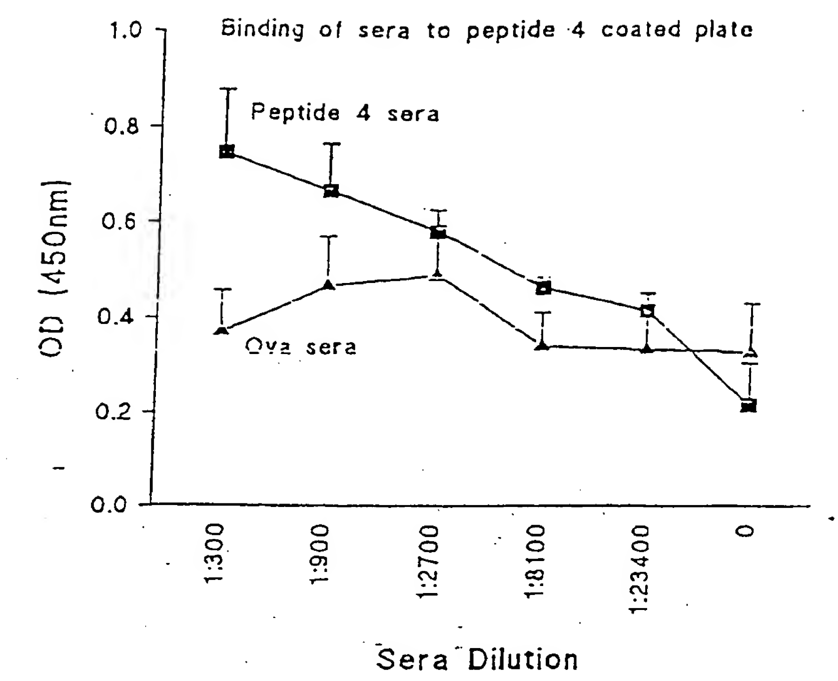


Figure14b Sera from mice immunised with peptides 4 and 6 recognise the peptide sequence in vitro.

Sera were harvested at day 35 (Figure 3) from peptide sensitised mice. The sera were diluted in ELISA buffer and titrated onto peptide coated plates. Plates were incubated for 1 hour at 37^{0}C. After washing, biotinylated sheep anti-mouse IgG (1:8000) was added and plates incubated as described above. After washing, Streptavidin-HRP (1:4000) was added for 1 hour at 37^{0}C. Plates were washed three times prior to the addition of TMB substrate. The reaction was stopped after 8 mins with 1M sulphuric acid, at the OD measured at 450nm.



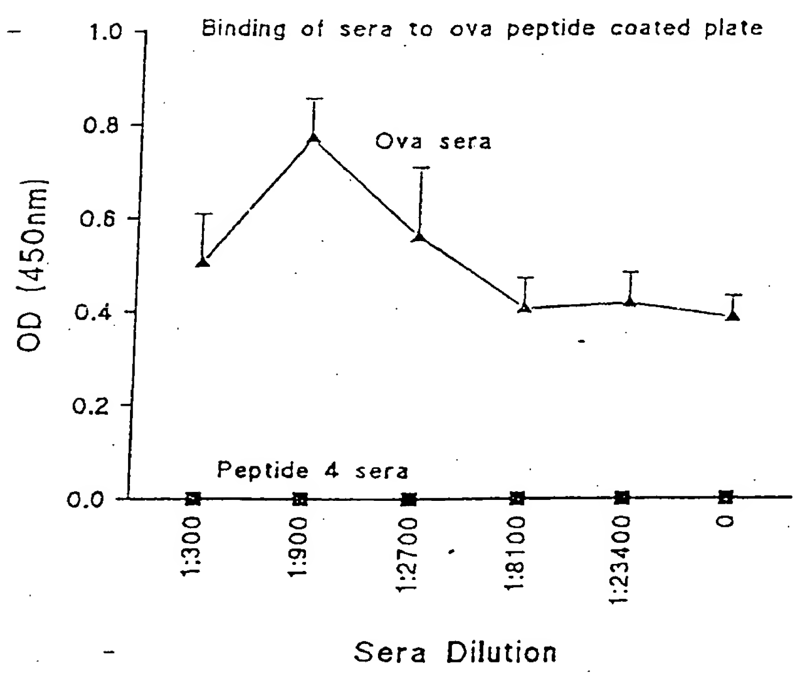


Figure 15a Differential binding of B7-2 specific sera or ova control sera as determined by Peptide ELISA. 96 well plates were pre-coated with either the B7-2 specific peptide Pep4. Ova control peptide (OVA) or no peptide. Sera harvested from peptide 4, or Ova peptide immunised mice were then screened for binding. Sera were detected by subsequent incubations with goat anti-mouse IgG-Biotin, Streptavidin-HRP and then developed with TMB. Plates were read at 450nm.: Values represent means +/- SEM for 4 mice per group, in duplicate wells. Values have been adjusted for binding to no-peptide control plate.Sera were measured over a range of dilutions.

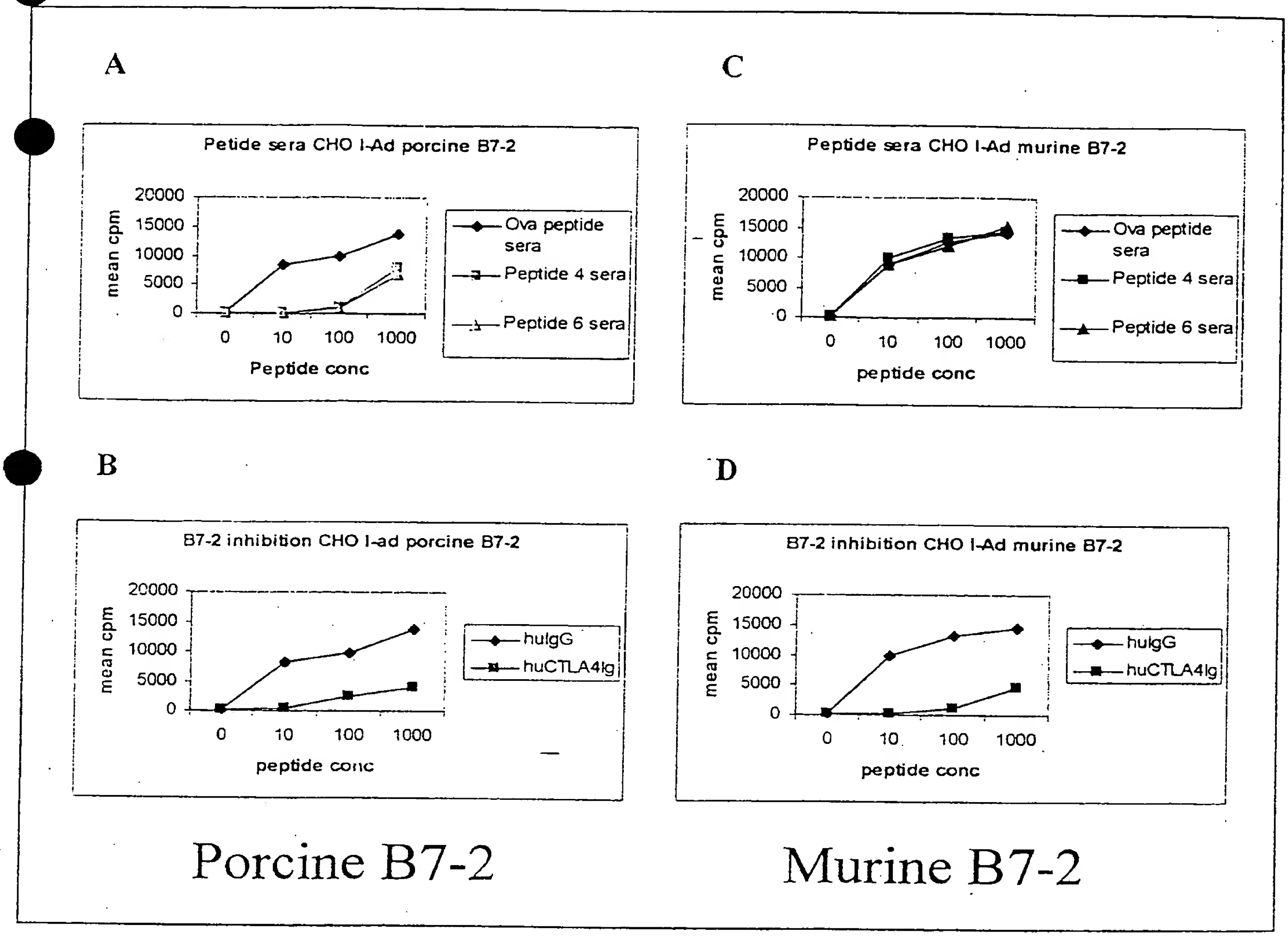
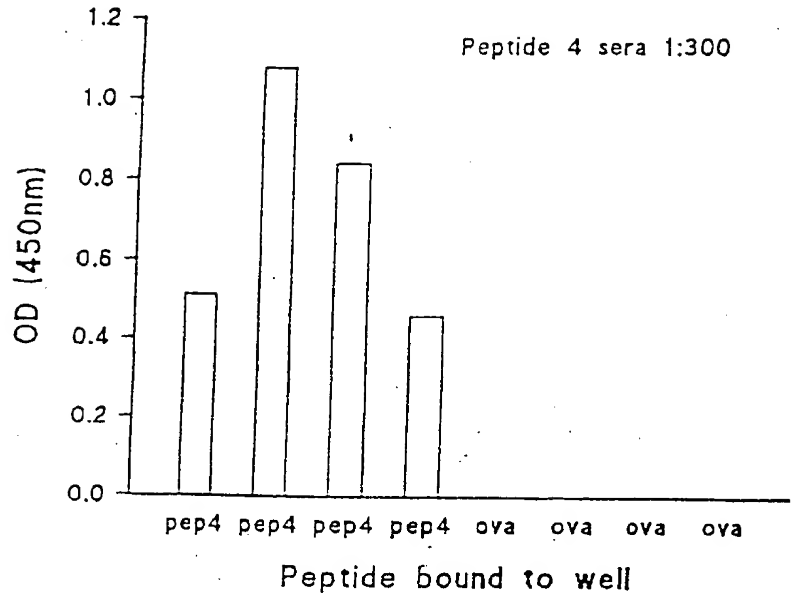


Figure 5k The anti-peptide antisera inhibit direct mouse anti-porcine T cell responses but have no effect on the delivery of costimulation by murine CD86.

T cells were purified from DO.11.10 T cell receptor transgenic mice which are restricted for OVA 323-339 in the context of the MHC Class II molecule I-A^d. 2 x10^4 T cells were cultured for 48 hours with CHO I-A^d stimulator cells transfected with either porcine CD86 (A-B) or murine CD86 (C-D). T cell proliferation was measured by the incorporation of thymidine over a 16 hour period. Sera from peptide 4 and 6 immunised mice inhibited T cell proliferation when costimulation was provided by porcine CD86 (A) but not murine CD86 (C).



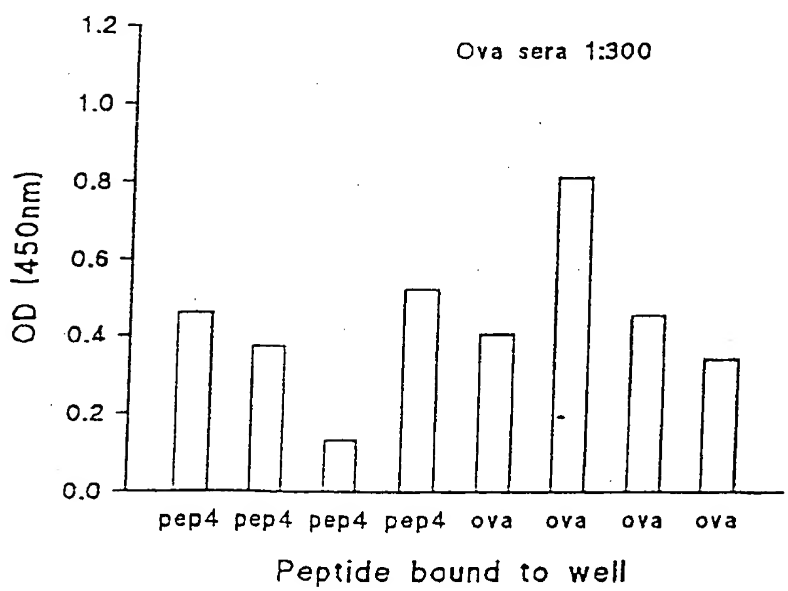


Figure 16 : Differential binding of B7-2 specific sera or ova control sera as determined by Peptide ELISA. 96 well plates were pre-coated with either the B7-2 specific peptide Pep4, Ova control peptide (OVA) or no peptide. Sera harvested from peptide 4, or Ova peptide immunised mice were then screened for binding. Sera were detected by subsequent incubations with goat anti-mouse IgG-Biotin, Streptavidin-HRP and then developed with TMB. Plates were read at 450nm. Values have been adjusted for binding to no-peptide control plates and represent means for duplicate wells for individual mice at 1:300 dilution of the sera.

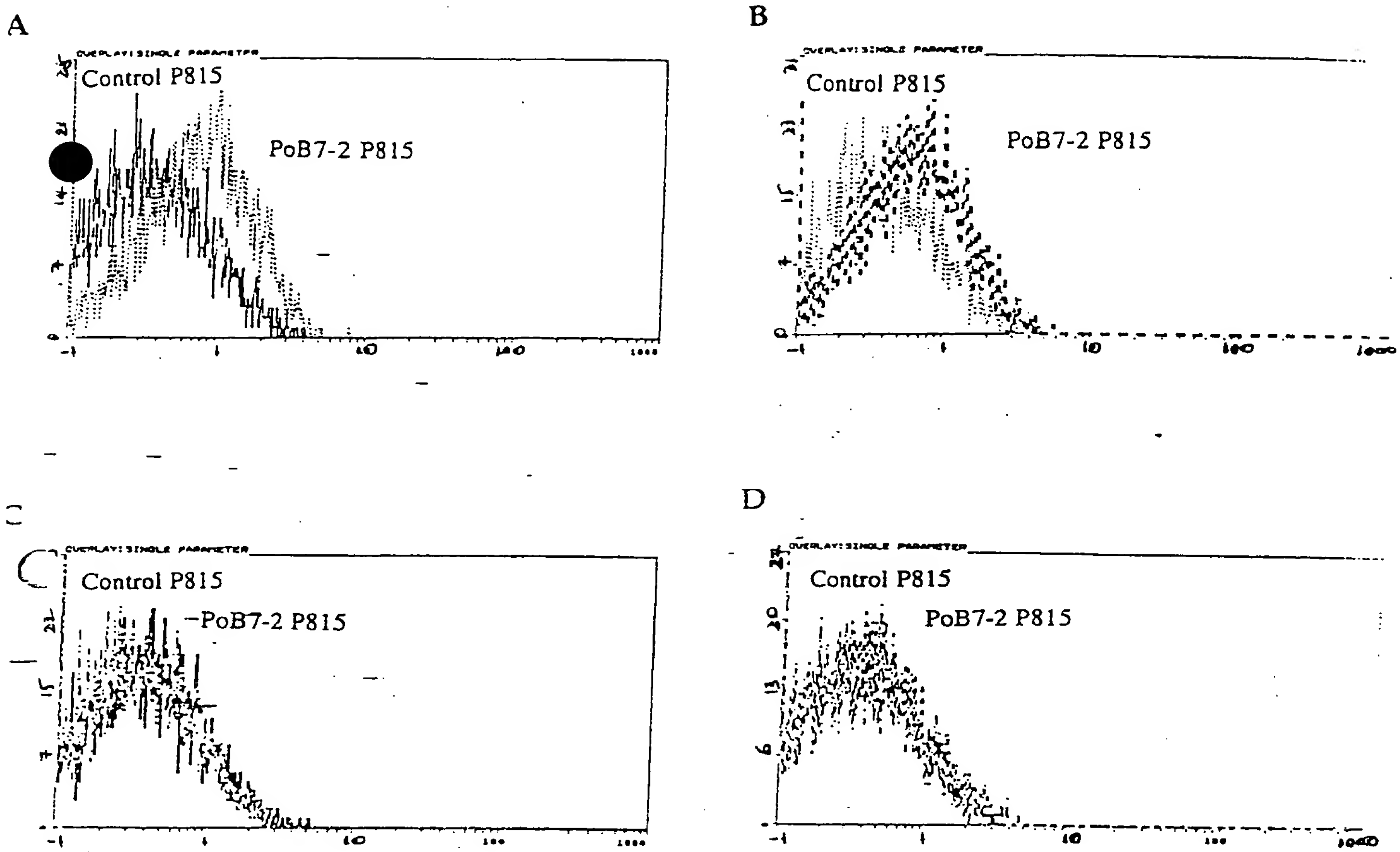


Figure 17a Flow cytometric analysis of porcine B7-2 transfected, or control untransfected P815 cells following staining with sera from peptide 4 or ovalbumin peptide control sera. 2.5 x 10^5 P815 cells were stained with 1μl of sera from 4 different mice immunised with either B7-2 peptide 4 (Figures A & B) or ova control peptide sera (Figures D & E). After washing, cells were incubated with goat anti-mouse IgG (H & L)-HRP and subsequently, Streptavidin-FITC. Cells were fixed with 1% paraformaldehyde and analysed on a Coulter counter.

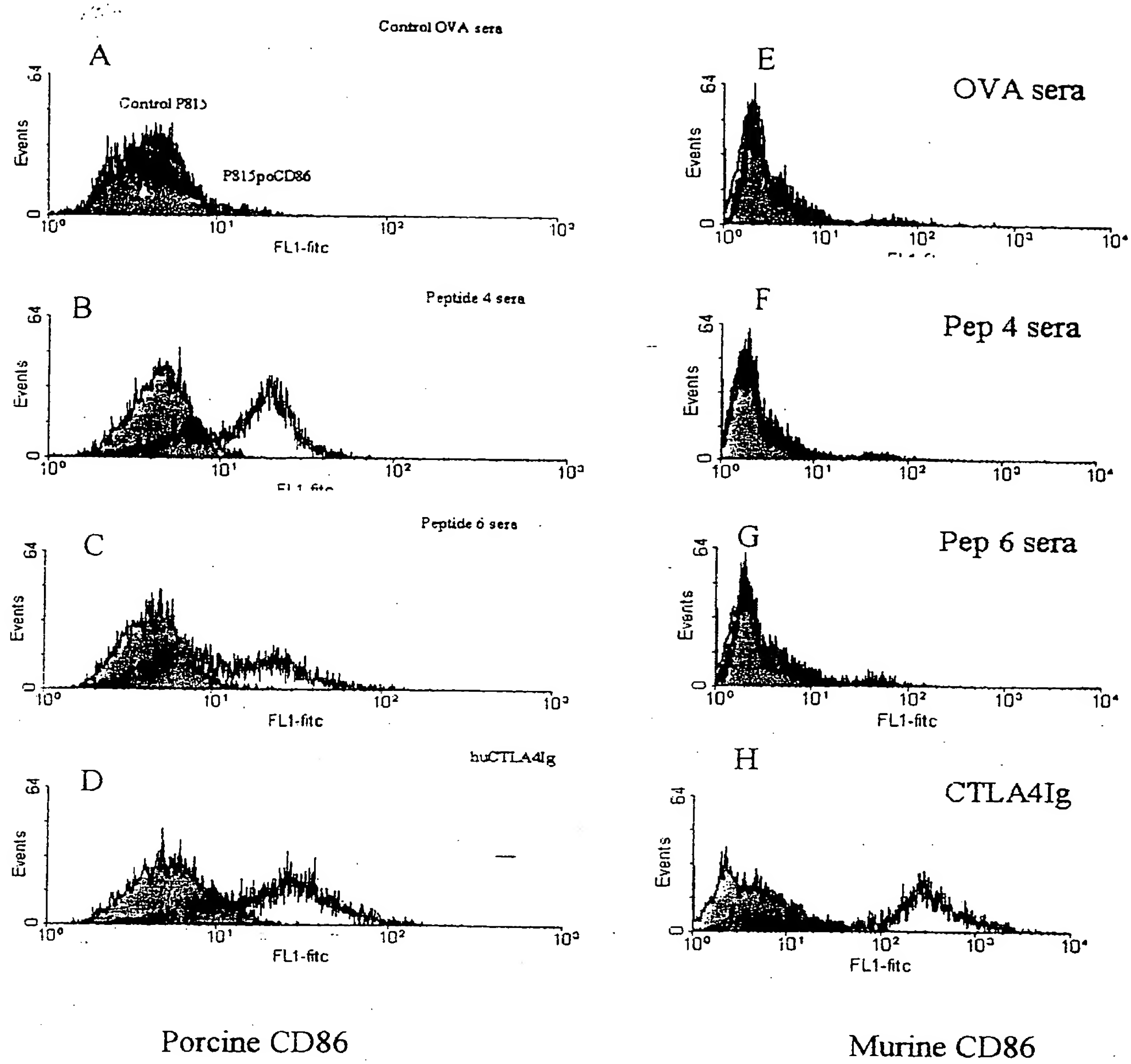
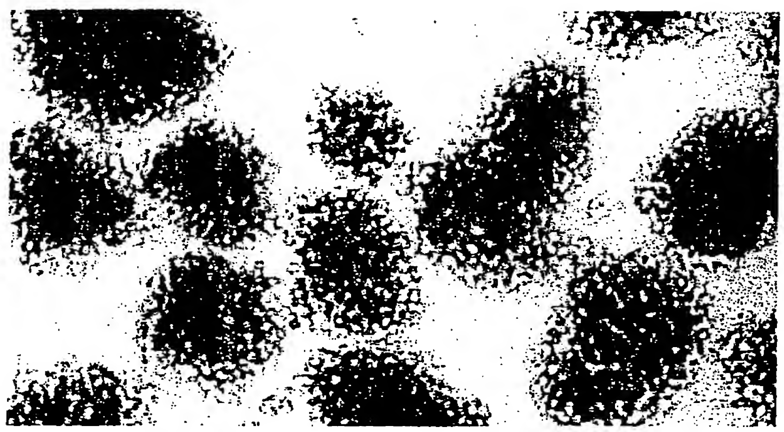


Figure 11b: Sera from peptide 4 and peptide 6 immunised mice recognise native porcine CD86 but not murine CD86 on transfected cells.
P815 cells transfected with porcine CD86 or CHO cells transfected with murine CD86 were stained with 1:25 dilution of sera harvested from peptide immunised mice (black line). Untransfected P815 and CHO cells were also stained for control purposes (red line) . Bound sera was detected by biotinylated sheep anti-mouse IgG (1:250), followed by streptavidin-FITC (1:100). 5000 cells per sample were analysed by flow cytometry using CellQuest software. Sera from peptide 4 and 6 immunised mice recognise porcine CD86 (A and B) but not murine CD86 (E and F). Sera from Ova peptide control mice do not recognise either molecule (C and G). CTLA4Ig staining of both cell lines confirms expression of CD86 on both the P815 and CHO transfectants (D and G).

Figure 18: Photograph of a preparation of porcine pancreatic islets purified from a large white pig.



Day 1: Immunisation of C57BL-6 mice with whole ovalbumin (50 micrograms) in Complete freunds adjuvant (CFA)

↓

Day 14: First immunisation with chimeric peptide (100 micrograms) i.v.

Day 21: Second immunisation with chimeric peptide (100 micrograms) i.v.

Day 28: Third immunisation with chimeric peptide (100 micrograms) i.v.

↓

Day 32: Mice rendered diabetic by injection of streptozotocin i.p.

↓

Day 36 : Transplantation of 1000 porcine pancreatic islets under the kidney capsule of diabetic mice

↓

Day 37 onwards : Survival of islets assessed by measuring blood glucose levels

Figure 19: Schematic representation of the chimeric peptide immunisation and transplantation protocol.

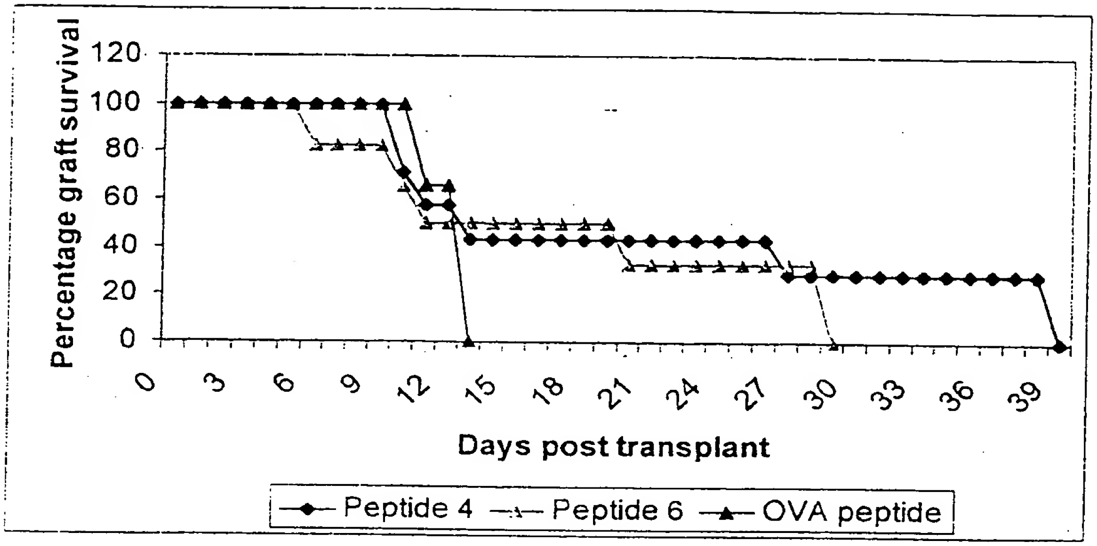


Figure 20 Anti-porcine CD86 antibody prolongs the survival of transplanted porcine pancreatic islets.

1000 islets are transplanted under the kidney capsule of C57BL-6 mice rendered diabetic by streptozotocin. Survival of the islets is determined by monitoring blood glucose using BM-144 strips. A blood glucose reading of 10 or above is considered as the onset of rejection.